TO PURIFY AND CHARACTERIZE METALLOTHIONEIN FROM HUMAN TISSUES, AND INVESTIGATE ITS ROLES IN MAMMALIAN COPPER METABOLISM AND IN THE MENKES KINKY HAIR DISEASE

By

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This dissertation is dedicated to my family, especially my parents and May-Fong, who have supported me with love and patience.

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Abstract of Dissertation Presented to the Graduate Council of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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In basal culture medium, intracellular copper concentration of MKHD fibroblasts was 310 ± 64.4 ng/mg cell protein compared to 108 ± 20.6 ng/mg cell protein in control normal cultures. The intracellular copper concentration of MKHD cells was twice that of normal fibroblasts at medium copper concentrations below 20 µg/ml. The 2 values approached each other at concentrations from 20 to 45 µg/ml. Death of MKHD cells occurred at medium copper concentrations between 15 and 20 µg/ml. The intracellular copper concentration was between 800 to 970 ng/mg cell protein. Normal cells died at medium copper concentrations above 30 µg/ml with an intracellular copper concentration above 1500 ng/mg cell protein. These observations suggested the presence of a regulatory mechanism for intracellular copper in normal fibroblasts at medium copper concentrations below 30 µg/ml which was defective in MKHD fibroblasts at all concentrations.

Intracellular cadmium concentration of MKHD cells was similar to that of normal cells (23.6±6.4 ng/mg cell protein versus 23.5±9.3 ng/mg cell protein) in basal medium, but was always higher at other medium cadmium concentrations (0.1 to 1.5 μg/ml) indicating an increased cadmium uptake by MKHD cells.

To clarify the mechanism of metal uptake in cultured fibroblasts, incorporation studies were done with both ^{64}Cu and ^{109}Cd at 37°C over a period of 10 hours. Uptake rate of ^{64}Cu in the first hour was about 3 times greater in MKHD cells than in normal cells, while that of ^{109}Cd was about the same in both types of cells. Subsequent uptake of both radionuclides was higher in MKHD cells than in normal cells.

Effluxes of ^{64}Cu and ^{109}Cd in both MKHD and normal cells were studied by pulse labelling the cells with the radionuclides for 20 hours and chased with non-radioactive medium. At both 37°C and 4°C, MKHD cells demonstrated a higher retention of both radionuclides than normal cells, indicating an impairment of efflux of both metals in MKHD cells. Lowering the temperature did not increase the percent retention of either radionuclides in the 2 types of cells, implying that the efflux processes are not energy requiring.

Inducibility of metallothioneins in MKHD cells by copper and cadmium was investigated utilizing L-(^{35}S) cysteine. ^{64}Cu failed, while ^{109}Cd succeeded in inducing metallothionein in both normal and MKHD fibroblasts. However, the metallothionein induced by cadmium in MKHD cells appeared to have a lower molecular weight than that in normal cells.

Partially purified metallothioneins (MT-1 and MT-2) from 1 MKHD and 2 normal livers were analyzed. MKHD metallothioneins demonstrated reduced cadmium, copper, zinc, and sulfhydryl group content.

Isotope exchange studies, carried out by incubating native metallothioneins with ^{64}Cu or ^{109}Cd, demonstrated a decreased affinity for copper and an increased affinity for cadmuim in both MKHD metallothioneins.

These observations imply an abnormality in MKHD fibroblast copper and cadmium transport which is associated with structural abnormalities in metallothioneins. A hypothesis is proposed in which metallothioneins function as a carrier by combining with the cations and carry them to the site of excretion. Abnormality in MKHD resides in the lower affinity for copper of the metallothioneins.

CHAPTER I

INTRODUCTION

General

There are a number of mineral elements present in minute amounts in living tissues. They occur in such a small quantity that early workers were unable to measure their precise concentrations with the analytical methods then available. Such elements were therefore described as "trace elements." Even though the importance of these trace elements to biological systems were known for a long time, remarkable advances in trace element research only occurred in recent years. This is mainly due to the development of exceedingly sensitive methods of analysis, especially atomic absorption spectroscopy and neutron activation.

There is no clear line of demarcation between the trace elements and the major elements. Elements that occur or function in living tissues in concentrations most conveniently expressed in µg/g or µg/l are generally considered trace elements (1).

Even though classification of the trace elements into essential, non-essential, and toxic groups can be inaccurate and misleading as all the essential elements become toxic at sufficiently high intakes, for the sake of convenience, Underwood (2) classified the trace elements into 3 groups: the dietary essentials, the possibly essentials, and the non-essentials. For an element to be considered as essential, it must fulfill the criteria postulated by Cotzias (3): the element is present in all healthy tissues of all organisms; its concentration in these is

relatively constant; its withdrawal from the body produces similar structural and physiological abnormalities in different species, which are prevented or reversed by addition of the element; and the abnormalities induced by deficiency are always accompanied by pertinent, specific biochemical changes, which can be prevented or cured when the deficiency is prevented or cured. At the present time 14 trace elements are believed to be essential for animal life. These are iron, copper, iodine, zinc, manganese, cobalt, molybdenum, selenium, chromium, nickel, tin, silicon, fluorine, and vanadium (1,4). The possibly essential elements include arsenic, barium, bromine, strontium, and boron (4). Some other 30 elements in addition to the foregoing are present in tissues in highly variable concentrations. These include elements such as aluminium, antimony, mercury, cadmium, germanium, gold, silver, lead, rubidium, bismuth, titanium, etc. Among these elements, cadmium, mercury, lead, and in addition, arsenic are toxic at relatively low concentrations (2,4).

The most important functions of the essential elements are their necessity for a large number of enzymes and their association with nucleic acids. Trace elements may be an integral part of the protein molecule, e.g. in a metalloenzyme, or participate in the enzyme action. Mechanisms by which metals function in enzymes include direct participation in catalysis, combination with substrate to form a metal-substrate complex upon which the enzyme acts, formation of a metalloenzyme that binds substrates in an enzyme-metal-substrate (or enzyme-metal-coenzyme-substrate) complex, combination of metal with a reaction product to alter equilibrium, and maintenance of quaternary structure (5).

Distribution of Copper

Copper, as an essential trace element, plays a vital role in numerous biochemical and physiological functions in higher animals. There is

strict homeostatic control over the levels of copper in the body; excess, as well as deficiency, will cause malfunctioning of the body or even death.

The total body stores of copper in the normal adult human range from 75 to 120 mg (6-8). It is widely distributed throughout the body. Liver, having 6.63 µg copper/g wet weight, has the highest copper concentration (9), followed by brain (5.41 µg/g wet weight), heart, lung, intestine, spleen, muscle, and bone (10). The liver alone contains about 10 % of the total body copper, while muscle and bone, because of the large mass, contain approximately 50 % of the total (10). Newborn and very young animals are normally richer in copper per unit body weight than adults of the same species (11).

Physiological Roles of Copper

Copper is present as stable complexes and chelates with organic- and macro-molecules, such as amino acids, nucleic acids, proteins, etc., as well as being the necessary constituent of a number of metalloproteins. In the majority of enzymes which contain copper, the copper acts as cofactor.

The major enzymes in mammals for which copper is essential are ceruloplasmin, cytochrome oxidase, monoamine oxidase, superoxide dismutase, diamine oxidase, uricase, tyrosinase, ascorbic acid oxidase, and dopamine-β-hydroxylase (12,13). The major non-enzymatic copper containing metalloproteins include metallothionein, transferrin, and conalbumin (13).

Ceruloplasmin is an α globulin with a molecular weight of 160,000 daltons and 8 atoms of copper per molecule (14). It is a heterogeneous (15), multifunctional enzyme. It oxidizes aromatic diamines, especially p-phenylenediamine (16), as well as epinephrine, norepinephrine, serotonin, and melatonin. Osaki et al. (17) suggested that it may function in

controlling the plasma level of certain amines. It is also essential in promoting hematopoiesis in the sense that it catalyzes the oxidation of ferrous to ferric ion thereby promoting the transfer of iron from storage cell to plasma transferrin (18,19).

Cytochrome oxidase catalyzes the oxidation of reduced cytochrome c by molecular oxygen. Each monomer of cytochrome oxidase contains one molecule of heme and one atom each of iron and copper (20). The molecular weight of cytochrome oxidase, however, is not known.

Superoxide dismutase has been referred to as haemocuprein, hepatocuprein (21), erythrocuprein (22,23), and cerebrocuprein (24). It has a molecular weight of 34,000 daltons and contains 2 atoms of copper and 2 atoms of zinc per molecule (25). This protein catalyzes the dismutation of superoxide free radical anions and thus has a vital role in protecting the cell from damaging effects of the superoxide radical (26,27).

There are a variety of monoamine oxidases in mammals. The molecular weight of the enzymes is 120,000 daltons, and has 1 atom of copper per molecule. The main function of these enzymes is to catalyze the oxidative deamination of monoamines to form the corresponding aldehydes (28). Lysyl oxidase catalyzes the oxidative deamination of peptidyl lysine to form peptidyl 2-aminoadipic-δ-semialdehyde, the precursor of desmosine, which forms cross-links in elastin and collagen, the structural proteins of elastic, bone, and connective tissues (29-31). Thus, monoamine oxidase is important in maintaining the structural integrity of both vascular and bone tissues.

Tyrosinase is responsible for catalyzing the first 2 steps in the synthesis of melanin pigment from tyrosine (32) and is therefore important in the pigmentation process. The molecular weight and the stoichiometry of copper in the enzyme are not known.

Copper and Disease

The most common method of detecting abnormal deviation of copper metabolism in human disease is by studying the copper level in blood and urine of the patients. The known abnormalities in copper metabolism have been divided into 2 categories according to blood copper level, namely hypocupremia and hypercupremia (33).

Disorders associated with hypocupremia may be due to the inability to synthesize ceruloplasmin, or excess loss or destruction of ceruloplasmin. In the former case, there are 3 conditions, namely: conditioned copper deficiency; failure to absorb copper because of sprue or coeliac disease, or inability to synthesize other copper-carrying proteins such as in the newborn, Wilson's disease (hepatolenticular degeneration), kwashiorkor, and idiopathic hypoproteinemia. In the case of excess loss or destruction of ceruloplasmin, there are 2 possibilities. The first is loss through excretion such as in nephrotic syndrome, or protein-losing enteropathy. The second is the accelerated catabolism of copper (33).

Disorders associated with hypercupremia are frequent. They include pregnancy, viral and bacterial infection, myocardial infarction, lupus erythematosis, hemochromatosis, biliary cirrhosis, Hodgkin's disease, leukemia, malignant disease, asplastic anemia, thyrotoxicosis, schizophrenia, and alcoholism (33).

Although copper is an essential trace element, excess of it is fatally toxic. Acute copper poisoning in humans results in ulcerations of the intestinal mucosa, hepatic cell necrosis, nausea, vomiting, diarrhea, hemoglobinuria, and jaundice (34). Chronic copper poisoning in animals results in hepatic and renal cell degeneration, hemoglobinuria, jaundice, and early death (35).

A large number of disorders have been associated with copper deficiency. They include anemia, depressed growth, bone disorders, abnormal hair growth, depigmentation of hair, neonatal ataxia, impaired reproductive performance, heart failure, cardiovascular defects, and gastrointestinal disturbances (2).

Copper is an essential component of adult red cells and is required for both the production and maintenance of their integrity in the circulation. Copper deficiency results in impaired iron metabolism, which affects the synthesis of hemoglobin (33). In copper deficiency conditions, bones are characterized by abnormally thin cortices, deficient trabeculae, and wide epiphyses with frequent fractures and severe deformities. Collagen extracted from the copper deficient bones contains less aldehyde group and is more soluble than that form control bones. The primary defect is reduction in amine oxidase activity (2). Neonatal ataxia is due to a low copper content in the brain, leading to deficiency of cytochrome oxidase in the motor neurons. Depletion of cytochrome oxidase activity leads to inhibition of aerobic metabolism and causes impairment of phospholipid synthesis resulting in demyelination of the central nervous system (2). Copper is required for the formation or incorporation of disulfide groups in keratin synthesis. Lack of copper will interfere with the arrangement of polypeptide chains in keratin synthesis resulting in the formation of steely hair (12). Cardiac lesions in copper deficiency are related to a reduction of amine oxidase activity of the aorta. This reduction causes formation of less cross-links in elastin resulting in decreased elasticity and, therefore, easier rupture of the aorta (2).

Besides these established relationships between copper and pathology in diseases, there are two less established physiological and pathological

roles of copper. Recently, Klevay (36) found that hypercholesterolemia in rats is induced by an increase in the dietary ratio of zinc to copper ingested. This led her to hypothesize that coronary heart disease is predominantly due to an imbalance in regard to zinc and copper metabolism (37). Inverse relationships were observed between vitamin A and copper levels in humans during pregnancy, with fevers, and in nephrosis. Moore (38) suggested that there may be some reciprocal interplay between Vitamin A and copper in their metabolism and mode of action.

Copper Metabolism

The minimum daily requirement of copper for the adult man is stated to be 1.5 to 2.0 mg per day (39). Infants require 0.04 to 0.14 mg per kg body weight per day (40). Ingested copper is poorly absorbed in most species (41). In normal human subjects, orally administered copper is absorbed to the extent of 32 % (8,42). Copper is absorbed both as ionic copper or in the form of complexes (6,10,43-47). There are no human data as to whether the cupric or cuprous form is more readily absorbed (48).

In most mammals copper absorption is limited to the stomach and small intestine. Following oral administration of radioactive copper to humans the activity appears very rapidly in the blood suggesting that copper absorption occurs in the stomach or upper small intestine, or both (49,50). In rat, copper is also absorbed in the stomach and duodenum (51), while in hamster, maximal copper absorption is observed in the lower middle section of the small intestine (52).

Dietary copper absorbed from the gastrointestinal tract is transported by the blood to various tissues. This can be shown by the rapid increase in plasma radioactivity after ingestion of radioactive copper (53-55). During the early phases following oral or intravenous administration, an

albumin-copper complex is identifiable as the transport vehicle for copper absorption (53,56). Although several binding sites are available on the albumin molecule, bovine serum albumin (57,58), human serum albumin (59,60), and rat serum albumin (61) each binds one copper ion preferentially prior to subsequent complexing with other copper ions. Serum copper is also transported by binding to amino acids, mainly histidine, glutamine, and threonine (62,63). These complexes, by virtue of their small molecular size, may facilitate the transport of copper across cell membranes (64-66). The amino acid-bound fraction of copper is in equilibrium with the albumin-bound copper, both of which are in equilibrium with ionic copper (59). Orally, as well as intravenously, administered radioactive copper disappears rapidly from the blood (53,67), followed by the occurrence of maximal concentration in the liver. Thereafter, a secondary increase occurs in plasma copper, accompanying the discharge of ceruloplasmin from the liver (53). Ceruloplasmin has been demonstrated in several experiments to be the donor of copper to other tissues (67-69). Copper in ceruloplasmin does not exchange with non-ceruloplasmin copper in vivo, suggesting the transfer of copper from ceruloplasmin to tissues involves a degradative mechanism (70-72).

It has been estimated that 2 to 5 mg of copper are ingested daily by adult man, 0.6 to 1.6 mg is absorbed, 0.5 to 1.3 mg is excreted in the bile, and 0.01 to 0.06 mg appears in the urine (8,42). Thus bile is the major pathway for excretion of copper, constituting the major portion of fecal copper (73).

Gitlin et al. (74) first demonstrated in mice that the amount of copper absorbed depends on the oral dose and absorption does not result exclusively from simple diffusion. Crampton et al. (52), using everted

sacs of hamster intestine, found that a small fraction of copper is transported from the mucosal side to the serosal side by an energy-dependent mechanism of limited capacity. The major portion of copper is taken up at a site either in or on the intestinal mucosa and subsequently released to the serosal side over a period of hours. Marceau et al. (75), using in vivo experiments in the rat, were able to demonstrate that the rate of absorption of copper increases linearly to a certain level and then decreases and finally reaches a constant level.

Amino acids are reported to facilitate transport of copper across cellular membranes from a variety of tissues (64-66,76). The rate of copper absorption also depends on the type of amino acids, its configuration, and the degree of polymerization (2,77). Since amino acid transport across mucosal membranes is known to be energy-dependent (10), the limited, energy-dependent mechanism for copper transport can be accounted for by the formation of copper-amino acid complexes. The more important passive mechanism, however, is postulated to be mediated by a protein-carrier.

Van Campen (78,79) demonstrated that both zinc and cadmium severely antagonize the intestinal uptake of copper in rats. The site of antagonism is demonstrated to be in or on the intestinal mucosa (80). Starcher (81) then identified a metal-binding protein of molecular weight 10,000 daltons in chick duodenum and suggested that cadmium and zinc antagonize copper absorption by competing for binding sites on that protein. Similarly, when radioactive copper and zinc are administered orally to rats, both isotopes can be found associated with a protein of 10,000 daltons molecular weight in the duodenum (82,83). Subsequently, Evans et al. (84) isolated the metal-binding protein from bovine duodenum and liver

and demonstrated that cadmium and zinc antagonize copper metabolism by displacing copper from the sulfhydryl binding sites of the protein. They suggested that the metal-binding proteins occurring in chick duodenum, rate organs, and bovine duodenum and liver are similar to metallothionein, a metalloprotein first isolated from equine kidney by Kägi and Vallee (85).

High dietary ascorbic acid increases the severity of copper deficiency in chickens (86,87) and rabbits (88). When high levels of ascorbic acid and radioactive copper were put into a ligated segment of rat intestine, copper absorption was significantly depressed. Whole-body retention studies demonstrated that the depressing effect of ascorbic acid upon copper retention is achieved primarily by reduced intestinal absorption (89). Based upon the fact that ascorbic acid decreases the binding of copper by metallothionein from both intestine and liver by inhibiting the formation of mercaptides, Evans et al. (84) suggested that metallothionein is the protein participating in the passive mechanism of copper absorption. Intestinal metallothionein provides binding sites within the intestinal mucosa for the removal of the metal from the dietary source and temporary storage for subsequent absorption.

Wilson's disease is an inborn error of metabolism affecting copper homeostasis (90). The patients suffer from the deleterious effects of excess tissue copper. The symptoms include excessive accumulation of copper in liver, brain, kidneys, and cornea, but with low serum ceruloplasmin levels, elevated non-ceruloplasmin-bound copper, increased urinary copper, and decreased fecal copper. As the disease progresses, the most significant observations are the slowing down of copper clearance from blood and the hepatic uptake of copper, as well as the gradual increase of non-ceruloplasmin plasma copper and urinary copper (10).

Evans et al. (91) were able to demonstrate that metallothionein from livers of patients with Wilson's disease has an abnormally high copper affinity.

Metallothionein

Copper is distributed throughout the hepatic subcellular fractions. However, the major portion of the total hepatic copper after differential centrifugation is found in the cytosol associating with a protein similar to metallothionein (82,92).

Metallothionein has been isolated from various sources, including the livers of human (93), equine (94), rabbit (95), rat (96), chicken (97), and mice (98), as well as the kidneys of rat (99), human (100), and equine (101). Molecular weight of the crude protein is about 10,000 to 12,000 daltons, while the purified form is about 6,600 daltons. The metal content may vary somewhat with different preparations, but is always close to 8 % and is higher than that of any other known metalloprotein. The metals present include cadmium (5-6 %), zinc (2-3 %), copper (0.3-0.5 %), and sometimes traces of mercury. Metallothionein is the only biological material known to contain cadmium (13). The protein also has a high sulfur content most of which is accounted for by cysteine (constitutes 20-30 % of its amino acid residues). The metals are bound to the protein by the sulfhydryl groups and roughly 3 mercapto ligands interact with one metal atom. The protein has no aromatic amino acid residues, and consequently has very low absorption at 280 nm. The cadmium mercaptide, on the other hand, is a chromophore with an absorption maximum at 250 nm (85,102). Polymorphism of metallothionein has been reported for the human and horse renal protein (100,101), human hepatic protein (93), and rabbit hepatic protein (103). The 2 forms of metallothionein have slight

differences in composition of amino acid residues, relative proportion of cadmium, zinc, and copper, pI values, and electrical mobilities in polyacrylamide gel. Metallothioncin prepared from various sources have roughly the same overall properties. However, the relative quantity of metal varies. The amino acid compositions also show some variation. The protein as a whole is acidic with a pI between 3.9 and 4.5 (103). It carries 2 negative charges at pH between 7 and 9. In liver, metallothionein amounts to 1 to 2 % of the total soluble proteins. Webb (104) suggested that metallothionein may be organ- as well as species-specific. However, no proof has been provided. The metal ions of metallothionein can be removed at low pH, and the metal-thionein can be reconstituted by adding the corresponding metal ions to the apo-protein (97,105). The apo-protein carries 4 positive charges at neutral pH (106).

The primary structure of one species of equine renal metallothionein was recently reported (106). The sequence shows distinct clustering of the 20 cysteinyl residues into 7 groups separated by stretches of at least 3 other residues. Within these groups the cysteines occur 7 times in alternating Cys-X-Cys sequences and 3 times each in Cys-Cys and Cys-X-X-Cys sequences, where X is an amino acid other than cysteine. Another feature is the close association of serine and of the basic amino acids with cysteine. These features demonstrate the presence of structural defined metal-binding sites which contain 3 cysteinyl residues as the principle metal-ligands forming a negatively charged trimercaptide complex $(Metal^{2+}(Cys^{-})_3)^{-}$. The basic amino acids and serine may also possibly be involved in metal binding. Equine renal metallothioneins and human, rabbit (106, 107), and mice hepatic metallothioneins (108) all have acetylmethionine as the amino terminus and alanine as the carboxyl terminus.

Metallothioneins are inducible metalloproteins. Cadmium is especially efficient in inducing the protein. Cadmium has been shown to be able to induce the synthesis of metallothionein in livers of rats (95,96, 109-118), rabbits (119), mice (117,118,120), and chicken (109), rat kidney (114,117,118), rat intestine (121), as well as in cultured human skin epithelial cells (122) and pig kidney cells (123). Zinc is able to induce metallothionein in rat liver (109,110,124-126), and rat intestine (126-128). Copper has also been reported to induce metallothionein synthesis (104,129-131), though there are controversies on whether the protein induced in the copper-loaded rat is metallothionein or not (132-134). In addition to cadmium, zinc, and copper, only mercury (135,136) is known to induce the synthesis of the corresponding metallothionein. The induced metallothioneins have the inducing metal as the major metal constituent. However, cadmium, zinc, and copper are always present though in varied proportions. The half-life of the induced protein is about 4.2 days in rats (131).

The molecular mechanism of metallothionein induction has only been investigated briefly. Bremner and Davies (130) found that cycloheximide when injected simultaneously with copper into rat, virtually abolishes amino acid incorporation into hepatic metallothionein, and also inhibits, by at least 90 %, the amount of copper appearing in the protein, implying that the induction mechanism involves translation. Richards and Cousins (137), on the other hand, showed that in rats actinomycin D, administered parenterally, inhibits zinc uptake into liver cytosol and metallothionein synthesis, implying that synthesis of the protein is controlled at the transcriptional level by the plasma zinc concentration. No further definitive evidence has been published.

Various roles have been suggested for metallothionein. These include the detoxification of heavy metals such as cadmium and mercury (95,96,100, 104,115,116,120,129,138-140), the metabolism of essential trace metals such as zinc (104,128) and copper (104,141), storage and transport of metals (10,81,104,141), and the intestinal absorption of copper and zinc (10,142).

Menkes Kinky Hair Disease

Menkes kinky hair disease (MKHD; also called Menkes kinky hair syndrome, Menkes steely hair disease, and trichopoliodystrophy) was first described by Menkes et al. (143) in 1962. It is a progressive central nervous system degenerative disorder transmitted as an X-linked recessive trait. It occurs in male infants. The onset of the recognizable symptoms of the disease is between the age of 4 to 8 weeks (144).

The patients have distinctive facies with pallid skin, pudgy tissues, horizontal tangled eyebrows, and a "cupid's bow" upper lip. The hair is hypopigmented, and pili torti can be seen under the microscope (145). Premature birth is frequent, and retardation of growth occurs in some cases (145). Other clinical manifestations include severe mental retardation, progressive cerebral degeneration, frequent convulsions, hypothermia, instability of temperature control, skeletal changes with Wormian bones of the skull and metaphyseal spurs of the femurs, tortuosity and elongation of blood vessels and death before 3 years of age. Necropsy findings in the brain include extensive neuronal degeneration, gliosis, and cystic degeneration (145-153).

The X-linked recessive inheritant pattern is well documented (144). Some heterozygous females show mild pili torti (145,154) and in one female sib of a black infant with MKHD, the skin pigment was also variegated (154).

Total number of published cases was 37 in 1976 (141). The frequency of the disease is estimated to be 1 in 35,000 live births in Victoria of Australia (155).

Symptoms similar to that in MKHD have also been observed in copper deficient animals such as sheep, rats, and chicken (156-161). Copper deficiency can explain all the clinical features of the disease as described previously under the title Copper and Disease.

MKHD patients have very low levels of serum copper (25 µg compared with 114 µg/100 ml serum in normal)(162), serum ceruloplasmin (10 mg compared with 33 mg/100 ml serum in normal)(162), liver copper (9 µg compared with 34 µg/g dry tissue in normal)(163), and brain copper (12.5 µg compared with 50 µg/g dry tissue in normal)(163). Oral administration of copper produces no significant clinical or biochemical changes, while intravenous administration of copper in form of copper-albumin complex is successful in raising the serum copper and ceruloplasmin levels to normal (162,164). Danks et al. (145) postulated that the basic biochemical defect of MKHD is defective intestinal absorption of copper. Studies of the duodenal biopsies of MKHD patients reveal abnormally high mucosal copper levels (60 ppm dry weight compare with 18.5 ppm dry weight in normal), indicating that the defect in copper absorption lies in the transport within the mucosal and epithelial cells of the gut or across the membrane on the serosal aspect of these cells (155,165). Dekaban et al. (166,167), using copper-67 (^{67}Cu), demonstrated that patients with MKHD absorb 11-13 % of ^{67}Cu given orally, compared with 45 % by unaffected controls. Though intravenously administered copper is retained 2 to 3 times longer by the patient's liver, it is less readily available for the synthesis of ceruloplasmin (167). A greater fraction of intravenous dose

is also found to be excreted than would have been predicted from the studies in normals and in patients with Wilson's disease (162). These results indicate defective utilization of intravenous copper. Normal infants do not develop symptoms of copper deficiency even if they were placed on copper-free diet for 60 days (168). The fact that MKHD patients may develop symptoms at 4 weeks of age suggests that they are copper-depleted at birth. This in utero copper deprivation may be due to defective maternal-fetal copper transport (162). All these observations can be explained by the hypothesis that the disease is due to an absence or abnormality of metallothionein (162).

MKHD was recently studied using cultured fibroblasts from the patients and also amniotic fluid cells of pregancies at risk. Cultured MKHD fibroblasts always show a higher intracellular copper concentration than normal cultures. The initial uptake rate of copper is also higher in MKHD cultures (169,170). Horn (171), using radioactive copper, was able to show higher incorporation of copper in cultured MKHD fibroblasts and amniotic fluid cells than normals. These observations appear to imply an increased affinity for copper in MKHD cells (170,171).

Rationale

In view of the previous discussion, it can be seen that the normal mechanism of intestinal copper absorption may be best studied by contrasting the MKHD patient with the normal. Due to the fact that MKHD is relatively rare, a direct study on the patient, or on post-mortem tissues is difficult. However, expression of the abnormal gene in cultured cells has been suggested by the demonstration of metachromasia in fibroblasts cultured from skin of patients and of heterozygotes (165). These observations suggested that cultured MKHD fibroblasts might serve as an in vitro model for studying the apparent abnormality of copper metabolism

occurring in patients. Additionally, prenatal disturbances in copper metabolism have been reported in a male fetus at risk for MKHD (172,173). Thus, establishment of cultured cells as a model for studying MKHD may make prenatal diagnosis of the disease possible utilizing cultured amniotic fluid cells.

With the assumption that metallothionein is responsible for intracellular transport of copper, and that the basic defect in MKHD resides in the transport of copper within the mucosal cells or across the membrane on the serosal side of cells, a comparative study of the dose response to copper and cadmium of cultured MKHD and normal fibroblasts should reveal the basic differences in handling metals. Since metallothionein appears to play a role in regulating cytotoxicity of metals (96,100,129, 138), the toxic levels of copper and cadmium should be different in MKHD and normal cultures.

Uptake of copper from the intestinal lumen into the mucosal cell was reported to be normal in MKHD patients (155,174). If the basic defect lies in the release of the cation by mucosal cells, a study of uptake and retention of metal ions by the cultured cells should clarify the differences between the mutant and the normal.

Copper and cadmium have been reported to induce metallothionein synthesis. Though the basic biochemical defect of MKHD was postulated to be in the intracellular transport of cations, the possibility of mutation in the inducibility of metallothionein cannot be excluded. The characteristic high cysteine content of metallothionein enables the utilization of L-(^{35}S) cysteine as an indicator of metallothionein de novo synthesis.

The most direct way to answer the question postulated for this investigation is to isolate metallothionein from post-mortem normal and MKHD

patient tissues. A comparative study of the amino acid content, metal content, as well as metal ion binding ability between the normal and the mutant will clarify the basic biochemical defect of MKHD.

CHAPTER II

MATERIALS AND METHODS

Cell Culture

Skin fibroblast cultures were established from biopsies of typical Menkes kinky hair disease (MKHD) patients using methods previously described (175). Additional MKHD fibroblast cultures were obtained from the Human Genetic Mutant Cell Repository (GM; Institute for Medical Research) and the American Type Culture Collection. Control cell cultures were also obtained from both the Human Genetic Mutant Cell Repository and the American Type Culture Collection. All the control cultures were derived from individuals that were comparable in sex and age to the MKHD patients.

All the cultures were grown in 12 ml completed media in 75 cm^2 plastic culture flasks (Corning) and incubated in a humidified environment of 5 % CO_2 at 37°C provided by a Wedco incubator. Completed media consisted of Eagle's minimum essential medium with Hank's balanced salt solution (HBSS; GIBCO) supplemented with 10 % fetal calf serum (ISI), 1 % antibiotic-antimycotic solution (ISI), and adjusted to a pH of 7.2 with 7.5 % sodium bicarbonate solution (ISI).

About 7 days were required for the cells to grow to confluent monolayers. Confluent cultures were subcultured in a 1:3 ratio by trypsinization at room temperature (176). Medium was changed every 3 days. Only confluent cultures with a passage number between 6 to 16 were used for experiments.

Reagent and Glassware

Reagent grade chemicals and double deionized water (Continental) were used throughout the study. Polyethylene labware were used whenever possible. All glassware and labware were cleaned by soaking in 50 % nitric acid for 3 hours and rinsed in deionized water (177). Aqueous solutions of all types were analyzed for copper, cadmium, and zinc before use and purified by passage through ion exchange resin if necessary (178).

Metal Analysis

Metal analysis was performed with a Perkin-Elmer model 306 Flameless Atomic Absorption Spectrophotometer equipped with a Perkin-Elmer model HGA-2100 Graphite Furnace. Five µl samples were used for each analysis. The times for drying, charring, and atomization of the samples were set at 15 seconds, 10 seconds, and 6 seconds, respectively. Copper (Cu) was analyzed at a wavelength of 324.7 nm with a slit width of 0.7 nm, charring temperature of 1,000^{o}C, and atomization temperature of 2,500^{o}C. The sensitivity of the machine for copper was 50 pg per 0.0044 absorption unit. Cadmium (Cd) was determined by absorbance at a wavelength of 228.8 nm with a slit width of 0.7 nm, charring temperature of 400^{o}C, and atomization temperature of 1,500^{o}C. The sensitivity of the machine was 2 pg per 0.0044 absorption unit. Zinc (Zn) could be determined at two different wavelengths depending on the concentration of the metal in the sample. For samples with low concentration, a wavelength of 213.9 nm was used. The sensitivity of the machine at this wavelength was 1 pg per 0.0044 absorption unit. For samples with a high zinc content, a wavelength of 307.6 nm was used. The sensitivity of the machine at this wavelength was 4,700 pg per 0.0044 absorption unit. For both wavelengths, the slit width, charring temperature, and atomization temperature were

0.7 nm, 500°C, and 2,000°C, respectively. Standard solutions were made by appropriate dilutions of the copper, cadmium, zinc 1,000 ppm reference standards (Fisher). Standards were run before and after each set of samples, and blank was run several times in the middle to assure stable base line.

Protein Determinations

The concentration of protein was determined either by the method of Lowry et al. (179) or, in case of presence of a large quantity of metallothionein, by the biuret reagent (180). In both cases bovine serum albumin (Sigma) was used as standard. The absorbance at 250 nm served as an alternative method for estimating the concentration of metallothionein. The absorptivity of a preparation of highly purified human renal metallothionein was determined to be 6.8 ml $mg^{-1}cm^{-1}$ at 250 nm (85). All the chemicals needed for protein assay were obtained from commercial suppliers and were of analytical or reagent grade.

DNA Assay

Cell Cultures in confluent monolayer were rinsed twice with Calcium-magnesium free Hank's balanced salt solution (CMF-HBSS; ISI). The cultures were then incubated with 2 ml of trypsin-EDTA 1X solution (ISI) at 37°C for 1 minute. Trypsin-EDTA solution was then withdrawn and the cultures were allowed to sit for another 5 minutes at 37°C. When the cells began to round up and detach, 5 ml of double deionized water were added to each flask to aspirate and to suspend the cells (181). Cells were lyzed by rapidly freezing and thawing the cell suspension twice in dry ice and acetone. The lysate was centrifuged at 10,000 xg for 20 minutes at 4°C in an automatic refrigerated centrifuge with a fixed angle rotor (Sorvall type RC2-B). The supernatant was saved for assaying metals and protein.

The pellet was resuspended in 200 µl of double deionized water and kept cold at 4°C. An equal volume of 0.4 M perchloric acid was added to precipitate nucleic acids and proteins. After 30 minutes at 4°C, the precipitate was pelleted by centrifugation at 10,000 xg for 10 minutes and the supernatant was removed (182). The pellet was hydrolyzed with 2 ml of 1.5 N perchloric acid at 37°C for 30 minutes. After cooling, 4 ml of 4 % diphenylamine reagent (183) with 0.01 % paraldehyde (184) were added. The assay tubes were sealed and incubated at 37°C overnight (182). The tubes were then centrifuged at 10,000 xg for 10 minutes to pellet undissolved protein and the absorbance of the supernatant was measured at 600 nm against a reagent blank. Standards were obtained by dilution of a stock solution of highly polymerized calf-thymus DNA (Sigma) in 5 % perchloric acid.

Radionuclide Counting

Radionuclides used included copper-64 (^{64}Cu), cadmium-109 (^{109}Cd) and sulfur-35 (^{35}S). ^{64}Cu was purchased from New England Nuclear in the form of carrier-free $^{64}Cu(NO_3)_2$ in 1 N HNO_3. It has a half life of 12.9 hours and a decay energy of 1.677 Mev (185). Samples containing ^{64}Cu were counted in polypropylene test-tubes (Packard) with a Packard Auto-Gamma Counter model 5210 with the following settings: Full range 1 Mev, lower level 460, and window 103. The counting efficiency for this radionuclide was found to be 28 %. All counts were corrected for decay, employing the formula $N=Noe^{-\lambda t}$ where N is the number of unstable nuclei remaining after time t, No is the original number of unstable nuclei (time zero) and λ is the decay constant (186).

^{109}Cd was purchased from Amersham/Searle, in the form of carrier-free $^{109}CdCl_2$ in 0.1 M HCl. The half life of ^{109}Cd is 450.15 days, and

the decay energy is 0.16 Mev (185). The ^{109}Cd containing samples were also counted with the Packard Auto-Gamma Counter model 5210 in polypropylene test tubes. The settings were: Full range 0.25 Mev, lower level 32, and window 112. The counting efficiency of the machine for this radionuclide was found to be 36 %. Due to its relatively longer half life, ^{109}Cd counts were not corrected for decay.

Sterilized L-(^{35}S) cysteine hydrochloride was obtained from Amersham/ Searle in aqueous solution. ^{35}S has a half life of 87.2 days and a maximum beta emission energy of 0.167 Mev (185). Samples containing L-(^{35}S) cysteine were counted in a Packard Tri-carb Liquid Scintillation Spectrometer model 3320 after thorough mixing with 10 ml of Aquasol (New England Nuclear) in glass scintillation vials (Wheaton). The counting efficiency obtained was 92 %.

Interference of ^{109}Cd in liquid scintillation counting of ^{35}S and ^{35}S in gamma counting of ^{109}Cd were determined. It was found that 9.8 % of the gamma counts of ^{109}Cd appeared in liquid scintillation counting and 17.3 % of the liquid scintillation counts of ^{35}S appeared in gamma counting. There was no interference between ^{64}Cu counting and either ^{109}Cd or ^{35}S due to the big difference in their decay energies.

Polyacrylamide Gel Electrophoresis (PAGE)

Preparation of Gels

Acrylamide (Sigma) and N,N'-methylene-bis-acrylamide (bis; Sigma) were purified by recrystallization from chloroform and acetone, respectively.

To make the running gel, 3 solutions were required. These were a 3 M Tris buffer, pH 8.9 containing 0.6 % N,N,N',N'-tetramethylethylene-diamine (TEMED: Canalco), a running gel stock of 50 % w/v acrylamide and

5 % w/v bis aqueous solution, and a 0.14 % w/v aqueous solution of ammonium persulfate (Sigma). The ammonium persulfate solution is stable for only 7 days and was kept refrigerated. The running gel was made by mixing 1 part of Tris buffer, 2 parts of running gel stock solution, 4 parts of ammonium persulfate solution and 1 part of water. Polymerization of this mixture resulted in a 12.5 % polyacrylamide gel with 0.075 % TEMED and 0.07 % ammonium persulfate, pH 8.9 (187).

The stacking gel was made from 4 solutions: a 0.5 M Tris buffer pH 6.7 containing 0.6 % TEMED, a stacking gel stock solution of 19 % w/v acrylamide and 0.4 % w/v bis, a 40 % w/v aqueous sucrose (Sigma) solution, and a 0.004 % w/v riboflavin aqueous solution (Roche). The riboflavin solution was made fresh each time and stored in the dark. The stacking gel was made by mixing 1 part of Tris buffer, 2 parts of stacking gel stock, 1 part of riboflavin solution, and 4 parts of sucrose solution. Polymerization of this mixture gave a 4.75 % polyacrylamide gel at a pH of 6.7, with 0.075 % TEMED (188).

Both the running and stacking gel solutions were mixed and deairated. Glass tubes (5 mm I.D. X 120 mm) (Bio-Rad) precoated with column coat (Canalco) were filled up to 8.5 cm with running gel and the stacking gel was added carefully to the top of the running gel for 1.5 cm. Water was layered on top of the gel and the gel was allowed to photopolymerize for an hour.

Electrophoresis

Samples containing 50 to 100 µg of protein were mixed in a sucrose solution with a small amount of tracking dye (bromophenol blue; Sigma). The electrophoretic buffer contained 0.05 M Tris and 0.5 M glycine, pH 8.3. Samples were layered on top of the gels under the buffer. Electrophoresis

was performed using a Canalco model 1400 constant current power source. A current of 2-1/2 mA per gel tube was employed until the sample reached the running gel. The current was then increased to 5 mA per tube (189).

Staning for 1 hour in 0.125 % Coomassie blue (Eastman)-10 % acetic acid-40 % ethanol staining solution was followed by destaining in 10 % acetic acid-40 % ethanol destaining solution. Destaining was completed by changing the destaining solution 2 to 3 times over a 24 hour period.

Scanning of Gels

Immediately after destaining, gels were scanned at 550 nm with a Gilford Spectrophotometer model 240 equipped with a recorder. The scanning speed was set at 1 inch/minute.

Slicing and Dissolution of Gels

After destaining, gels were frozen in dry ice. Gels were cut into 1 mm thick slices with a gel slicer (Bio-Rad). Gel slices were dissolved by incubating 1 slice with 0.5 ml of 30 % hydrogen peroxide (Fisher) at 50°C for 4 hours in a glass vial (190).

Protein Sulfhydryl Determination

Sulfhydryl content of proteins was determined by utilizing the Ellman's reaction (191). Ellman's reagent was made by dissolving 40 mg of 5,5'-dithiobis 2-nitrobenzoic acid (DTNB; Aldrich) in 10 ml of 0.1 M sodium phosphate buffer, pH 8. About 0.005 to 0.02 µmole of protein was dissolved in 3 ml of 0.08 M sodium phosphate buffer, pH 8, containing 2 % w/v sodium dodecyl sulfate (SDS; Sigma) and 0.05 % w/v of ethylenediamine tetraacetic acid (EDTA; Sigma). To the protein solution 0.1 ml of DTNB solution was added. Color was developed for 15 minutes and read against a reagent blank at 412 nm. The concentration of sulfhydryl groups was calculated according to the formula $Co = \frac{A}{\varepsilon} D$ mole/$_1$ where Co was the original concentration of sulfhydryl group in M, A was the absorbance

at 412 nm, ε was the extinction coefficient of the color producing p-nitrothiophenol and was equal to 13,600 $M^{-1}cm^{-1}$, and D was the dilution factor of the protein (191).

Molecular Weight Estimation

Molecular weights were estimated by gel filtration (192), utilizing a 120 cm X 2.5 cm glass column (VWR Scientific) of either Sephadex G-75 (Sigma) equilibrated with 0.02 M Tris-0.005 M HCl, pH 8.6, or Bio-Gel P10 (Bio-Rad) equilibrated with 0.1 M ammonium bicarbonate, pH 8.2. Protein samples, 1 to 3 mg in 1 ml, were applied to the column and their elution volume estimated by spectrophotometric measurement. The void volumes were determined using Blue Dextran (M.W. 2 x 10^6; Pharmacia). Marker proteins employed included: ovalbumin (M.W. 46,000; Sigma), lysozyme (M.W. 13,930; Sigma), ribonuclease (M.W. 12,600; Sigma), ACTH (M.W. 4,200; Calbiochem), glucagon (M.W. 3,460; Calbiochem), and bacitracin (M.W. 1,411; Calbiochem).

Amino Acid Analysis

Amino acid analyses were carried out on samples oxidized with performic acid for 4 hours. The oxidized sample was hydrolysed with 6 N HCl for 24 hours in vials sealed under nitrogen at 110 ± 1°C in a Single-wall Transit Oven (Blue M Electrical) (193,194). Analyses were performed on a Durrum Amino Acid Analyzer model D-500 high pressure chromatograph using single column analyses. The steel column (48 cm x 0.175 cm) was packed with sulfonated benzene polystyrene resin (Durrum DC4A-8μ±1). Fluorescence detection employing o-phthalicdicarbonaldehyde (Aldrich) as the fluor was employed, allowing a sensitivity down to 30 to 50 pmoles.

Three sodium citrate buffers were used for elution of amino acids; 0.2 N, pH 3.25; 0.2 N, pH 4.25; 1.1 N, pH 7.9. Two temperature conditions were utilized. They were 50°C and 60°C. The temperature change to 65°C

was automatically programmed to occur with elution of alanine. Flow rate of buffer was 12.ml/hour. The pressure was 200 psi. A PDP8/M computer hooked up to the analyzer identified the amino acids and computed their concentrations automatically. Cysteine and methionine were determined using the calibration constants for cysteic acid and methionine sulfone, respectively. Values of serine and threonine were corrected for standard lost. Tyrosine and tryptophan were measured spectrophotometrically in the non-oxidized protein (195).

Dose Response of Culture Fibroblast to Metals

Copper sulfate ($CuSO_4$) solution were sterilized by filtering through Swinnex-25 filter units, 0.45 µm pore size (Millipore Corporation), added to the media of confluent cultures of control and MKHD cells to make copper concentrations of 2,4,6,10,15,20,25,30,35, and 40 µg/ml. The cultures were incubated for 48 hours at 37°C. After the medium was withdrawn, the culture was washed twice with 10 ml of CMF-HBSS.

Two ml of Trypsin-EDTA were used to trypsinize the cells and the rest of the harvesting procedure was as described under the DNA assay section. Copper content determination and protein assay were done on the supernatant after the first centrifugation and DNA assay was done on the pellet. Four ml of the supernatant were applied to a Sephadex G-75 superfine column (45 cm x 2.0 cm) equilibrated with 0.02 M Tris-0.005 M HCl, pH 8.6 at 4°C. Five ml fractions were collected with a flow rate of 24 ml/hour. The absorbance of each fraction at 250 nm and 280 nm were determined.

The above procedures were repeated using cadmium chloride ($CdCl_2$) solution. Effects of medium cadmium concentrations of 0.1, 0.3, 0.5, 0.7, 0.9, and 1.1 µg/ml were examined.

Metal Uptake Experiments

$^{64}Cu(NO_3)_2$ in 1N HNO_3 was adjusted to near neutrality with sodium bicarbonate solution and was sterilized by filtering through Swinnex-25 filter unit, 0.45 µm pore size. Seventeen µCi of ^{64}Cu were added to confluent cultures in 10 ml of media. The concentration of copper in the medium was adjusted to 9 µg/ml by addition of sterile $CuSO_4$ solution. The cultures were incubated at 37°C for 1, 3, 5, 7, and 10 hour periods. After withdrawing the radioactive medium the cells were washed with 5 ml of Calcium-magnesium free phosphate buffered saline twice and trypsinized. The radioactive medium, wash, and trypsin were pooled and 1 ml of the pool was counted for radioactivity. The cells were harvested with 2 ml of distilled water and the flask was rinsed twice with 1.5 ml of water. The 5 ml of aqueous cell suspension were pooled, and the cells were lysed. Protein content of the cell lysate was determined and 3.5 ml of the lysate were counted. The above experiment was repeated with the presence of media containing 0.09 µg Cd/ml.

The uptake of ^{109}Cd by cultured fibroblasts at 37°C in the absence and presence of copper were also examined. Four µCi of ^{109}Cd were added to cultures in 10 ml media, and the concentration of cadmium in the medium was adjusted to 0.56 ng/ml with $CdCl_2$ solution. Copper, if present, was of a concentration of 5 µg/ml medium. One ml of the medium, wash, trypsin pool, and 3.5 ml of the cell lysate were counted.

Competitive uptake of copper and cadmium was also examined by adding ^{64}Cu and ^{109}Cd to cultures simultaneously.

Metal Efflux Studies

Cultures were pulse labelled with 16.7 µCi of ^{64}Cu in 10 ml media with a copper concentration of 9 µg/ml at 37°C for 20 hours. After

rinsing twice with 5 ml of prewarmed media (37°C), the cultures were chased with 10 ml of prewarmed non-radioactive media. The chase procedures were done at 37°C and 4°C as well as in the presence of 0.09 µg/ml medium of cadmium at 37°C. Cultures were analyzed for ^{64}Cu and protein content at intervals of 1, 3, 5, 7, and 10 hours during the chase experiment.

The same set of experiments were repeated with ^{109}Cd. Radioactivity of ^{109}Cd in the pulse label experiment was 4 µCi/10 ml media, with a cadmium concentration of 0.06 µg/ml. Experiments with ^{64}Cu for pulse labelling and ^{109}Cd for chase, and vice versa, were also done. The quantity of radioactivity and the concentration of metal ions were the same as described above.

Metal Induction of Metallothionein

Capability of both copper and cadmium to induce the synthesis of metallothionein in both normal and MKHD cultured fibroblasts were examined by incubating the cultures in media containing both ^{64}Cu and L-(^{35}S) cysteine or ^{109}Cd and L-(^{35}S) cysteine at 37°C. The radioactive quantities added to 10 ml media were 34 µCi for ^{64}Cu, 4 µCi for ^{109}Cd, and 7.5 µCi for L-(^{35}S) cysteine which corresponded to concentrations of 5.5 µg/ml, 5.5 ng/ml and 0.2 nmole/ml of copper, cadmium, and cysteine, respectively. One ml of the cell lysate was counted for ^{35}S. Another ml was counted for ^{64}Cu or ^{109}Cd. Protein content was also determined. Electrophoresis was done with 0.2 ml of cell lysate. Instead of staining after electrophoresis, the gel columns were fixed in aqueous solution of 10 % acetic acid-40 % ethanol for 4 hours. The gels were then sliced, first counted for ^{64}Cu or ^{109}Cd and then dissolved and counted for ^{35}S. Control experiments were done by adding only L-(^{35}S) cysteine to cultures.

Preparation of Metallothionein

Control normal livers were obtained from autopsy specimens of adults as well as children of both sexes who died of causes other than diseases of the liver. MKHD patient liver was obtained from autopsy. All the tissues were stored frozen at -20°C. Unless otherwise stated, all steps for the preparation were carried out at 4°C. Before the preparation, the tissues were thawed for 15 to 18 hours at 4°C.

Due to the differences in the quantity of tissues available pure metallothionein was only prepared from control livers while a crude preparation of metallothionein was made from the MKHD liver.

Extraction Procedure

The preparation procedure was based on that described previously by Bühler and Kägi (93) with some modifications.

Livers were cut into small pieces and were homogenized in 1.5 volumes of 0.05 M potassium phosphate buffer, pH 7.0 in a Waring Blender for 20 seconds. The homogenate was stirred for 2 hours and centrifuged at 1,000 xg in a refrigerated centrifuge (Sorvall) for 60 minutes. The pellet was discarded. To the supernatant, 1.2 parts of 95 % ethanol and 0.095 part of chloroform, both prechilled at -20°C, were added dropwise. The resulted suspension was again centrifuged at 1,000 xg for 60 minutes. To the supernatant 2 parts of 95 % ethanol, prechilled to -20°C, were added to precipitate the metalloprotein fraction. The yellowish precipitate was collected by centrifugation at 3,000 xg for 20 minutes and reextracted for 10 minutes with 200 ml water. After removal of insoluble material by centrifugation at 20,000 xg for 2 hours, the supernatant was lyophilized.

Chromatographic Procedure

The lyophilized product was dissolved in 0.02 M Tris-0.005 M HCl, pH 8.6 and applied to a Sephadex G-75 column (120 cm X 2.5 cm), equilibrated with the same buffer. The material was eluted with this buffer at a flow rate of 30 ml/hour, and 20 ml fractions were collected. The eluted fractions were analyzed for cadmium content and the UV absorption at 250 nm and 280 nm were also measured. Fractions in which cadmium and 250 nm absorbance peaks coincided were pooled and applied to a DEAE Sephadex A-25 (Sigma) column.

DEAE Sephadex A-25 column (45 cm X 2.0 cm) was equilibrated with 0.02 M Tris-0.005 M HCl, pH 8.6. After sample application and washing with one column volume (600 ml) of the same buffer, the adsorbed material was eluted with a 1,600 ml linear salt gradient (800 ml 0.02 M Tris-0.005 M HCl, pH 8.6 and 800 ml 0.2 M Tris-0.05 M HCl, pH 8.6) and 600 ml limiting buffer. Constant flow rate of 30 ml/hour was maintained, and the effluent fractions, 14 ml each, were anayzed for cadmium and absorbance at 250 nm and 280 nm. Fractions in which the cadmium content and 250 nm absorption maxima coincided were pooled. Two fractions should be obtained. The first fraction came out before and the second fraction came out after application of the limiting buffer. The pooled fractions were desalted and concentrated by ultrafiltration at $4^{o}C$ in an Amicon pressure dialyzer equipped with an UM-2 membrane (Amicon).

The concentrated and desalted first fraction was applied to a DEAE Sephadex A-25 column (28 cm X 1 cm, equilibrated with 0.03 M Tris-0.0075 M HCl, pH 8.6), and eluted with the same buffer at a flow rate of 30 ml/hour, and 10 ml fractions were collected. The second fraction was applied to a DEAE Sephadex A-25 column (28 cm X 1 cm) equilibrated with 0.03 M Tris-

0.0075 M HCl, pH 8.6. Elution was accomplished with 1,000 ml of the same buffer followed by 1,600 ml of 0.06 M Tris-0.015 M HCl, pH 8.6. Ten ml fractions were collected. The metallothionein preparations obtained from these 2 columns were designated as metallothionein-1 (MT-1) and metallothionein-2 (MT-2), respectively.

In order to prepare crude MT-1 and MT-2 from MKHD livers, the last 2 DEAE-Sephadex A-25 columns were omitted. The 2 peak fractions obtained before and after the application of the limiting buffer were designated as MT-1 and MT-2.

Amino acid analysis was done on the pure metallothioneins, while PAGE, metal content, sulfhydryl content and biuret protein determinations were done on both pure and partially purified MT-1 and MT-2.

Metal-Binding Studies

Only partially purified MT-1 and MT-2 were used for these studies. The procedure was a modification of that described by Evans et al. (92). To a 25 ml conical flask containing 4 ml of 0.02 M Tris-0.005 M HCl, pH 8.6 were added 2.5×10^{-7} M of metallothionein and 3.78×10^{-10} M ^{109}Cd or 4.85×10^{-6} M ^{64}Cu. The solution was stirred continuously at 4°C for 2 hours. The solution was then dialysed for 24 hours in benzoylated dialysis tubing (Sigma) against 2 changes (1 l each) of deionized water. ^{64}Cu or ^{109}Cd and protein were determined in the dialysed solution.

The ^{109}Cd labelled metallothioneins were applied to a Bio-Gel P10 (Bio-Rad) column (45 cm X 2.0 cm) equilibrated with 0.1 M ammonium bicarbonate, pH 8.2. Fractions of 2.5 ml were collected, and ^{109}Cd was determined in each fraction.

Sulfhydryl Affinity Chromatography (Covalent Chromatography)

Preparation of Sulfhydryl column

To prepare hemissuccinate pantotheine, 2 g of L-pantotheine (Sigma)

were mixed in 50 ml chloroform and stirred gently. The pH of the mixture was maintained at 4.8 with 2 N sodium hydroxide. After 10 minutes the pH was brought down to 2.85 with 12 N HCl. The aqueous phase was separated and concentrated at 40°C using a flask evaporator; hemisuccinate of pantotheine was in the aqueous phase.

To prepare the sulfhydryl column, hemisuccinate pantotheine was reacted with an aminimide containing polymer treated with monoethanolamine (AID-EA, gift from Dr. John Tsibris). About 5.37 meq (2.5 g) of AID-EA were wetted with ethanol, filtered on a Büchner funnel and washed with water. Six ml of water were added to the polymer, and 0.054 mmole of hemisuccinate pantotheine and 0.159 mmole of a water soluble carbodiimide (Sigma) were added to the slurry which was stirred at room temperature for 50 hours. Then 1.90 ml of 1 M potassium phosphate, pH 7.4 were added, and the slurry was stirred for another 2 hours and filtered. The resin was washed with 40 ml water followed by 40 ml of ethanol and 3 volumes of 0.1 M Tris buffer, pH 8.0. Fifty ml of 20 mM dithioerythreitol (Sigma) were added, and stirred for 20 minutes, and then suction filtered. These two procedures were repeated thrice. The resin left on the filter was then washed with 500 ml of buffer until no dithioerythreitol could be detected in the filtrate with 2,2'-dipyridyl disulfide (Sigma) (196). One hundred ml of 2 mM 2,2'-dipyridyl disulfide were then added to the solid residue and the mixture stirred for 30 minutes. The reaction mixture was suction filtered, and washed with 0.1 M Tris buffer pH 8.0 until no absorption at 280 nm and 343 nm were detected in the filtrate. The residue was sequentially washed with 30 % methanol-70 % chloroform, 150 ml of ethanol and finally 150 ml of diethyl ether. The product was the "activated" pyridyl disulfide derivative of the aminimide polymer and was stored under nitrogen.

Preparation of apo-metallothionein

Liver was homogenized with 2 volumes of 0.05 M potassium phosphate, pH 7.0. The homogenate was stirred at 4°C for 2 hours and then centrifuged at 50,000 xg for 1 hour. The pellet was discarded. The supernatant was again centrifuged at 105,000 xg for 2 hours. The pellet was again discarded. From this point onwards, until the reaction with the pyridyl disulfide derivative of AID-EA, all the experiments were done in a glove box under nitrogen to prevent air oxidation of the sulfhydryl groups. The pH of the supernatant solution obtained above was adjusted to 2 with 2 N HCl under vigorous stirring. It was then centrifuged at 10,000 xg for 20 mintues. The supernatant was applied to a Chelex-100 (Bio-Rad) column (18 cm x 1 cm) equilibrated with 0.1 N HCl to remove all the displaced metal ions. The fraction collected was adjusted to pH 7 with sodium hydroxide and 2 M Tris buffer, pH 8.0. The apo-metallothionein solution thus obtained was stirred for 2 hours with a batch of prewetted AID-EA sulfhydryl derivative, removed from the nitrogen atmosphere and filtered. The resin recovered contained apo-metallothionein covalently bound.

Affinity Chromatography

The protein bound resin was washed several times with water, 100 ml of 2 M potassium chloride and water until no absorption at 280 nm was detected in the filtrate. Two ml of 0.4 M $CdCl_2$ solution were added to the resin which was stirred at 4°C for 3 hours after addition of 40 ml of water. The mixture was suction filtered, and the resin washed thoroughly with water until no 280 nm absorption was detected in the filtrate. Two nmole of 2-mercaptoethanol (Sigma) solution were added followed by 2 ml of 0.4 M $CdCl_2$ solution. The mixture was stirred for 2 hours, suction

filtered and washed with excess of water. All the filtrate was pooled, desalted, and concentrated by ultrafiltration in an Amicon pressure dialyzer equipped with an UM-2 membrane (Amicon). The overall procedure is outlined in Figure 1.

The concentrated filtrate was applied to a Sephadex G-10 (Sigma) column (45 cm x 2 cm) equilibrated with 0.02 M Tris-0.005 M HCl, pH 8.6 to remove excess metal ions. Cadmium content and 250 nm and 280 nm absorbance of the eluent fractions were measured. Fractions in which the peaks of the 3 quantities coincided were pooled, desalted and concentrated by ultrafiltration with an UM 2 membrane. Each of the concentrated fractions was applied to a Sephadex G-50 (Sigma) column (45 cm x 2 cm) equilibrated with the same buffer as before. Cadmium content and absorbances at 250 nm and 280 nm of each fractions were measured and those having the three peaks together were pooled, concentrated and desalted as before. Amino acid analysis was done on the concentrated fractions.

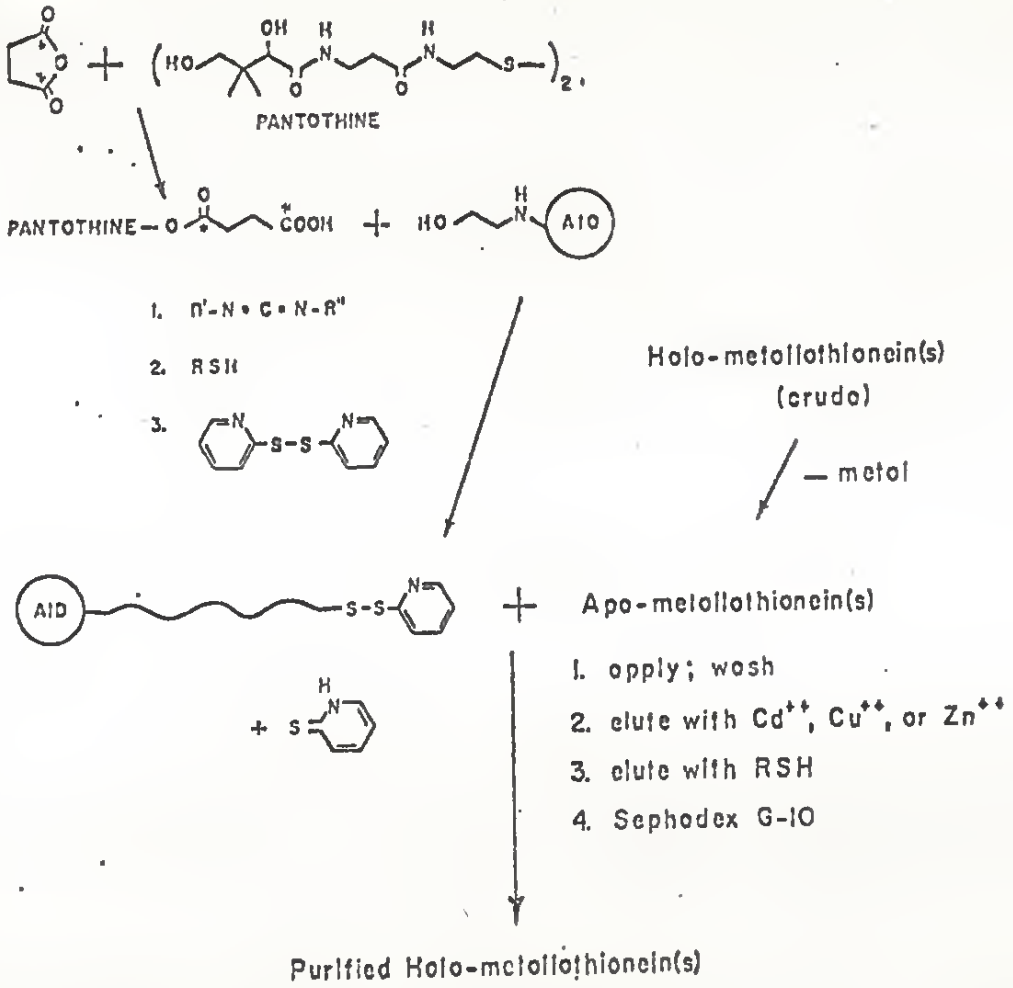


FIGURE 1. PREPARATION OF PURE HOLO-METALLOTHIONEIN USING SULFHYDRYL AFFINITY COLUMN CHROMATOGRAPHY

CHAPTER III

RESULTS AND DISCUSSION

Dose Response of Cultured Fibroblast to Metals

MKHD cultured fibroblasts were morphologically indistinguishable from normal cultured fibroblasts. When cultured normal and mutant cells were treated with media enriched with copper sulfate, no morphological change was observed at low medium copper concentration. MKHD cultured cells began to round up and detach from the bottom of the flask at medium copper concentrations of 15 to 20 µg/ml. These effects were interpreted to be due to the cytotoxicity of copper on the mutant cells. The morphological changes are shown in Figure 2. A cytotoxic change was observed with normal cultured fibroblasts at a significantly higher concentration of copper. Normal cells remained viable until the medium copper concentration was greater than 30 µg/ml.

This alteration in the copper cytotoxicity level may be due to either the sensitization of enzymes which include microsomal membrane ATPase (197,198), as well as a variety of glycolytic enzymes (199), towards copper, or a change in the homeostatic regulation of copper in the mutant cells. The latter mechanism seems more likely as implied from the findings in Wilson's disease where mutation of a gene (genes) is responsible for abnormal copper transport and excretion (90,200,201). Copper is toxic to organisms lacking barriers to absorption. The relative toxicity appears to be inversely proportional to the efficiency of the excretory mechanisms (200). Thus, higher life forms, e.g. avians, mammals, etc., have a much

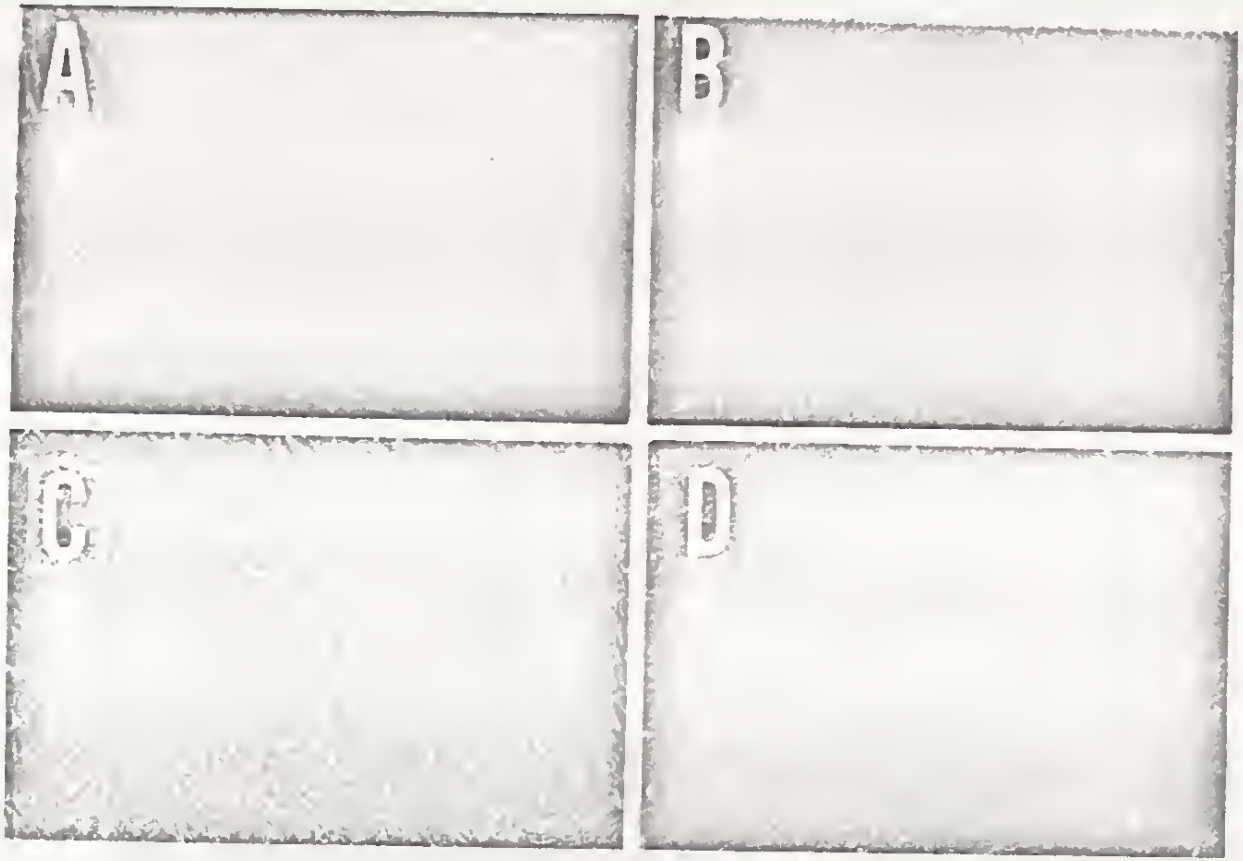


FIGURE 2. DOSE RESPONSE TO COPPER OF NORMAL CULTURED FIBROBLASTS

Cultured cells were incubated with media enriched with various quantities of copper for 48 hours. Morphology of cells was observed under a phase contrast inverted microscope (Leitz) with magnification of 100X

A. medium with 20 µg copper/ml
B. medium with 30 µg copper/ml
C. medium with 35 µg copper/ml
D. medium with 40 µg copper/ml

higher resistance to copper than lower life forms such as algae, insects, etc. The toxic level for fish, however, was noted to be similar to that for algae, plankton, insects, and invertebrates, apparently due to the fact that the gills of fish lack barriers to absorption and efficient excretory mechanisms for copper (200,202). Presumably, cultured cells have no barrier to absorption of copper (200). The decreased cytotoxic level of copper for the mutant cells might, therefore, be secondary to a less efficient excretory mechanism compared with normal.

To further investigate the differences in copper homeostasis between MKHD and normal cultures the copper content of the cultures was determined after they had been incubated with medium concentrations of copper varying from 2 µg/ml to 40 µg/ml. The results are presented in Table 1. In basal culture medium, the intracellular copper concentration of MKHD cells was 310 ng/mg cell protein compared with 108 ng/mg cell protein in normal cultures. This difference was maintained in all the MKHD cultures investigated as reflected by the small standard deviation. This observation is consistant with that reported by Goka, et al. (170). The MKHD cell results are similar but our data have a narrower range than reported by Goka, et al. The difference in the control values may be due to the fact that only age and sex matched normals were used in this investigation while the data reported (170) included cells obtained from subjects of varying donor age and/or sex. This increased intracellular copper content in MKHD cells agrees with the deduction from our cytotoxicity experiments, that MKHD cells have a less efficient excretory mechanism for copper than normal.

Intracellular copper concentrations in MKHD and normal cultures increased as a function of increasing medium copper concentration. How-

TABLE 1

COOPER CONTENT OF CULTURED FIBROBLASTS AT VARIOUS MEDIUM COPPER CONCENTRATIONS[a]

Medium Copper Conc. in µg/ml	0	1	4	6	10	15	20	25	30	35	40
MKHD cells (n=7)[b]	310 ± 64	605 ± 189	253 ± 46	264 ± 50	637 ± 229	804 ± 111	975 ± 331	1139 ± 195	1367 ± 332	2100 ± 1012	2222 ± 418
Normal cells (n=4)	108 ± 21	222 ± 74	106 ± 20	154 ± 37	259 ± 94	452 ± 133	1080 ± 151	1913 ± 608	1553 ± 383	2086 ± 516	2475 ± 720
Copper Content of MKHD cells / Copper Content of Normal cells	2.9	2.7	2.4	1.7	2.5	1.9	0.9	0.6	0.9	1.0	0.9

a. units shown are ng copper/mg cell protein; values are means ± standard deviations

b. n denotes number of different cultures tested

ever, at medium copper concentrations below 20 µg/ml the intracellular copper levels in MKHD culture were double that of normal. The ratio of MKHD to normal intracellular copper concentration approached unity when the medium levels exceeded 20 µg/ml. This would indicate that the homeostatic mechanism in MKHD cells was similar to that of normal cells overloaded with copper. MKHD cells began to die when the intracellular copper concentration was between 800 and 970 ng/mg cell protein which was about 3 times the base level, while death of normal cells occurred only at concentrations above 1,500 ng/mg cell protein (15 times the base level) indicating that MKHD cells have a decreased capacity to regulate copper. All these observations support the postulation of an inefficient excretory mechanism in MKHD cells.

To prove that these observations reflected an intrinsic defect in MKHD cells and not other factors which might affect intracellular copper concentrations, several control experiments were undertaken. The copper concentration of basal media and various culture solutions was determined. The average copper concentration of the media was 48.70 ± 20.0 ng/ml. Very little variation in copper concentration of normal and MKHD cultures were found with the medium copper concentration varying from 38 to 85 ng/ml (170). Thus, the difference in intracellular copper content between MKHD and normal in basal medium is a characteristic of MKHD cells.

Both MKHD and normal cultures required about 7 days to achieve confluency. To maintain similar culture morphology and characteristics at the time of experimentation only confluent cultures were analyzed. To investigate the validity of utilizing total cell protein as an estimate of culture size, the protein to DNA ratio was determined for both types of culture at various medium copper concentrations. Very little

variation was observed within the range of the copper concentrations studied. Furthermore, investigations by other groups utilizing total protein content and cell counts as estimates of culture size revealed little difference between MKHD and normal cultures (170). It was also reported that the copper concentration of normal cultures remained within the 95 % confidence limits for over 15 passages while the MKHD cultures seemed to possess a tendency to increase in copper content as the number of subcultures increased (170). To avoid the variation of results caused by passage number of cultures, all the cultures used had a passage number in the range of 6 to 13. Besides copper sulfate, copper acetate was also used as a source of copper ion. Similar results were obtained indicating that the sulfate ion did not affect the absorption and excretion of copper by the cells. The pH of the media used were tested after addition of copper sulfate and before and after incubation of the cultures at 37°C. The maximal change of medium pH after addition of copper sulfate was 0.3 pH unit. After incubation for 48 hours, the pH of all the cultures media was 6.6±0.3.

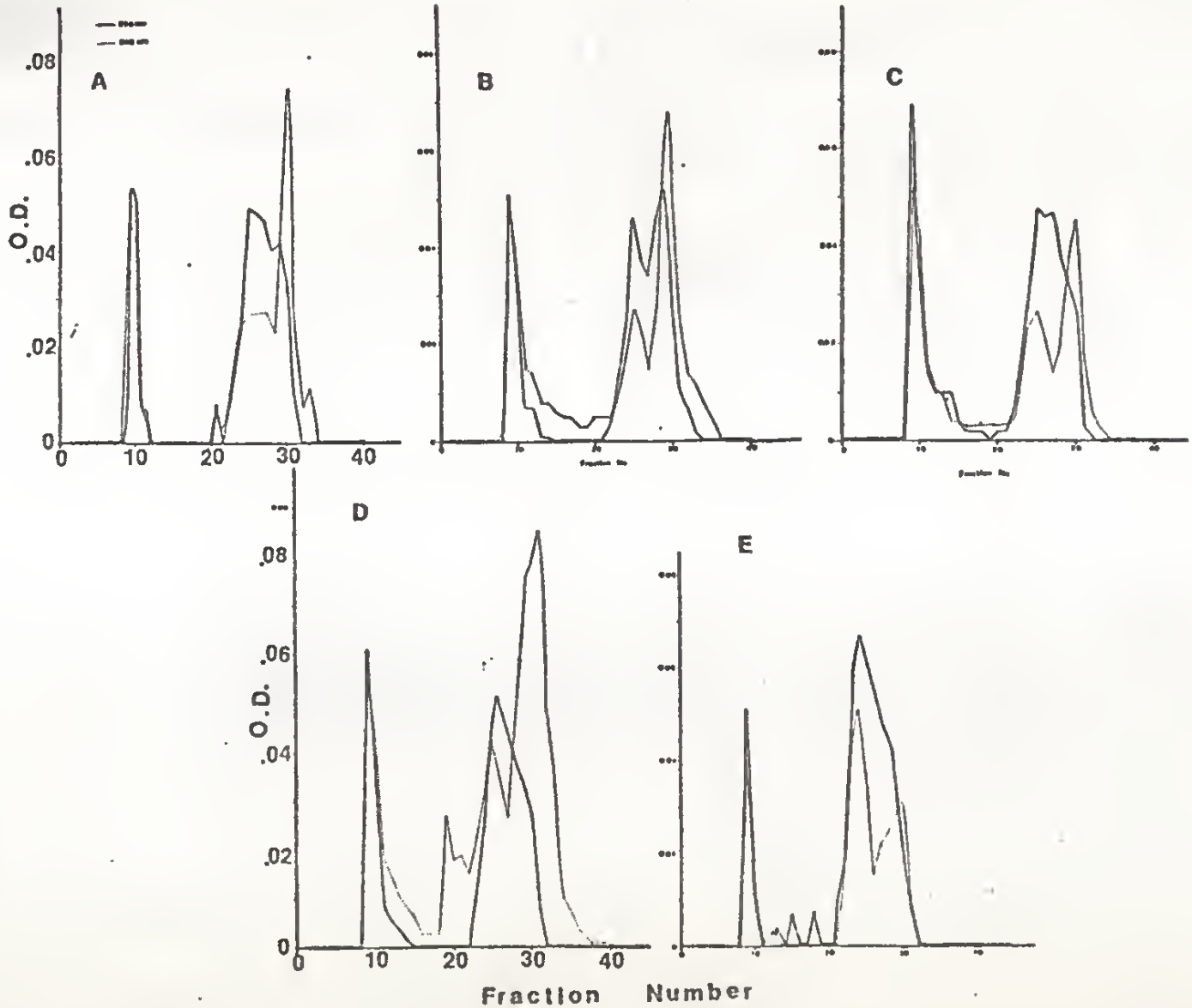
Sephadex column chromatography of cell lysates from copper treated MKHD and normal cells demonstrated a significant difference in the elution profile. The elution profiles of normal culture cell lysates at medium copper levels of 0,2,4,6,10 µg/ml are presented in Figure 3. Comparing the 250 nm absorption spectrum indicates disappearnace of a peak at fraction 30 as a function of increasing medium copper concentration. This fraction corresponded to a molecular weight of about 12,000 daltons. The peak became an inflection point at the medium copper concentration of 4 µg/ml. Similar chromatograms were observed with MKHD cells (Figure 4). However, the specific peak disappeared at a medium copper concentration of 2 µg/ml and at 8 µg/ml, even the shoulder disappeared.

FIGURE 3. SEPHADEX CHROMATOGRAPHY OF NORMAL CULTURED FIBROBLASTS AFTER TREATMENT WITH COPPER

Procedures was described under materials and methods ————250 nm absorbance

----------280 nm absorbance

A. in basal medium
B. in medium with copper concentration of 2 µg/ml
C. in medium with copper concentration of 4 µg/ml
D. in medium with copper concentration of 6 µg/ml
E. in medium with copper concentration of 10 µg/ml


A
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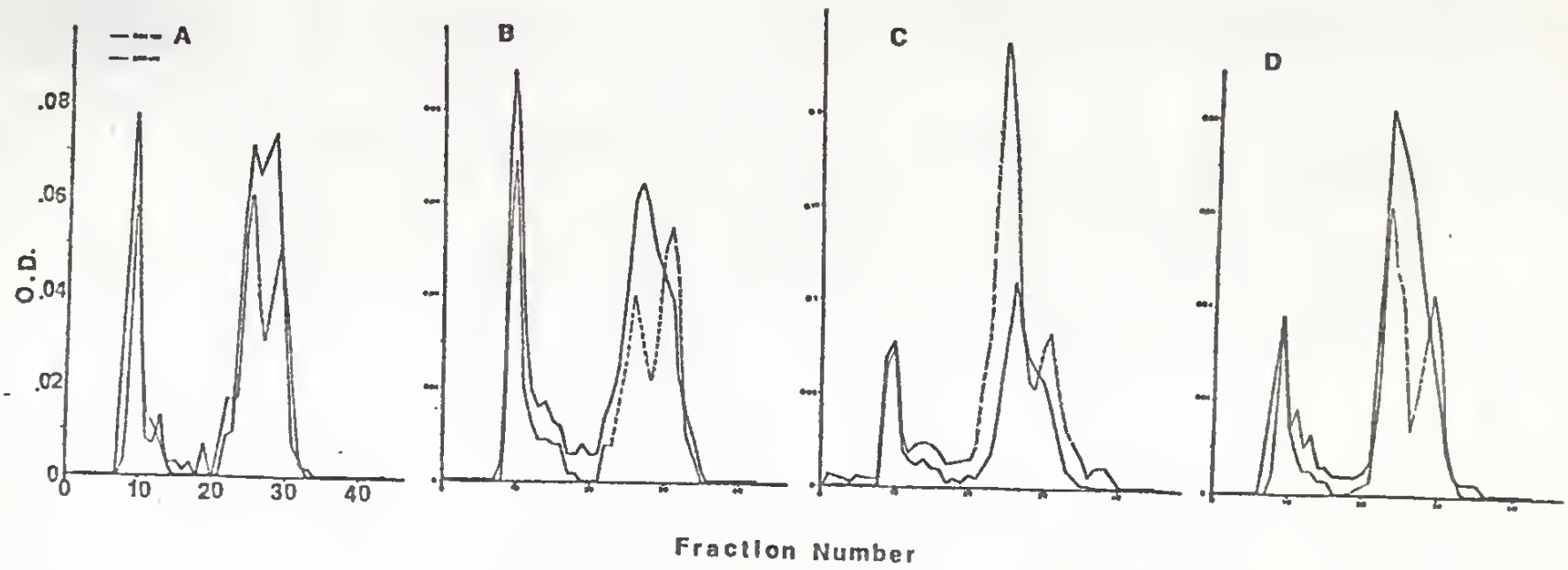


FIGURE 4. SEPHADEX CHROMATOGRAPHY OF MKHD CULTURED FIBROBLASTS AFTER TREATMENT WITH COPPER

Procedure was describedunder materials and methods ——————250 nm absorbance
----------280 nm absorbance

A. in basal medium
B. in medium with copper concentration of 2 µg/ml
C. in medium with copper concentration of 4 µg/ml
D. in medium with copper concentration of 8 µg/ml

Metallothionein in its crude form has a molecular weight of 10,000 daltons and exhibits maximum absorption at 250 nm due to the Cd-mercaptides in the protein (85). It is assumed that the disappearing peak corresponds physicochemically to metallothionein. Increasing the intracellular copper concentration resulted in displacement of cadmium from the Cd-mercaptide with a corresponding diminution in the 250 nm absorption. The peak disappeared at a lower medium copper concentration in MKHD cells probably because of its higher intracellular copper content than normal at the same medium copper concentration.

Both MKHD and normal cells were less resistant to cadmium than to copper toxicity. Cells rounded up and detached at medium cadmium concentrations below 0.5 µg/ml. The changes in morphology of cells upon intoxication with cadmium are shown in Figure 5. There was no significant difference in cytotoxic levels of cadmium between MKHD and normal cells. This toxic level for human fibroblasts was much lower than that for rat dermal fibroblasts which were killed at a medium cadmium concentration of 1.5 µg/ml (203), but was comparable to that for epithelial-like cells derived from adult pig skin (123). No cadmium was detected in basal culture medium. Both MKHD and normal cells showed an intracellular cadmium concentration of about 23.5 ng/mg cell protein, when incubated in basal medium. The intracellular cadmium concentration of MKHD cells increased more than that of normals at medium cadmium concentrations above 0.1 µg/ml, but was never more than double that of normals, except at a medium concentration of 0.3 µg/ml cadmium. Death of MKHD cell occurred at an intracellular cadmium concentration 2 to 3 times that for normal cells. The results are presented in Table 2. Confluent cultures were used throughout the experiment. Cultures tested had passage numbers between 8 to 13.

TABLE 2

CADMIUM CONTENT OF CULTURED FIBROBLASTS AT VARIOUS MEDIUM CADMIUM CONCENTRATIONS[a]

Medium Cadmium conc. in µg/ml	0	0.1	0.3	0.5	0.7	0.9	1.1
MKHD cells (n=5)[b]	23.6 ± 6.4	192 ± 42	866 ± 392	1165 ± 216	1073 ± 363	1434 ± 157	1018 ± 228
Normal cells (n=3)	23.5 ± 9.3	180 ± 84	241 ± 49	620 ± 207	765 ± 93	801 ± 113	655 ± 123
Cadmium content of MKHD cells / Cadmium content of Normal cells	1.0	1.1	3.6	2.0	1.4	1.8	1.5

a. units shown are ng cadmium/mg cell protein; values are means ± standard deviations

b. n denotes number of different cultures tested

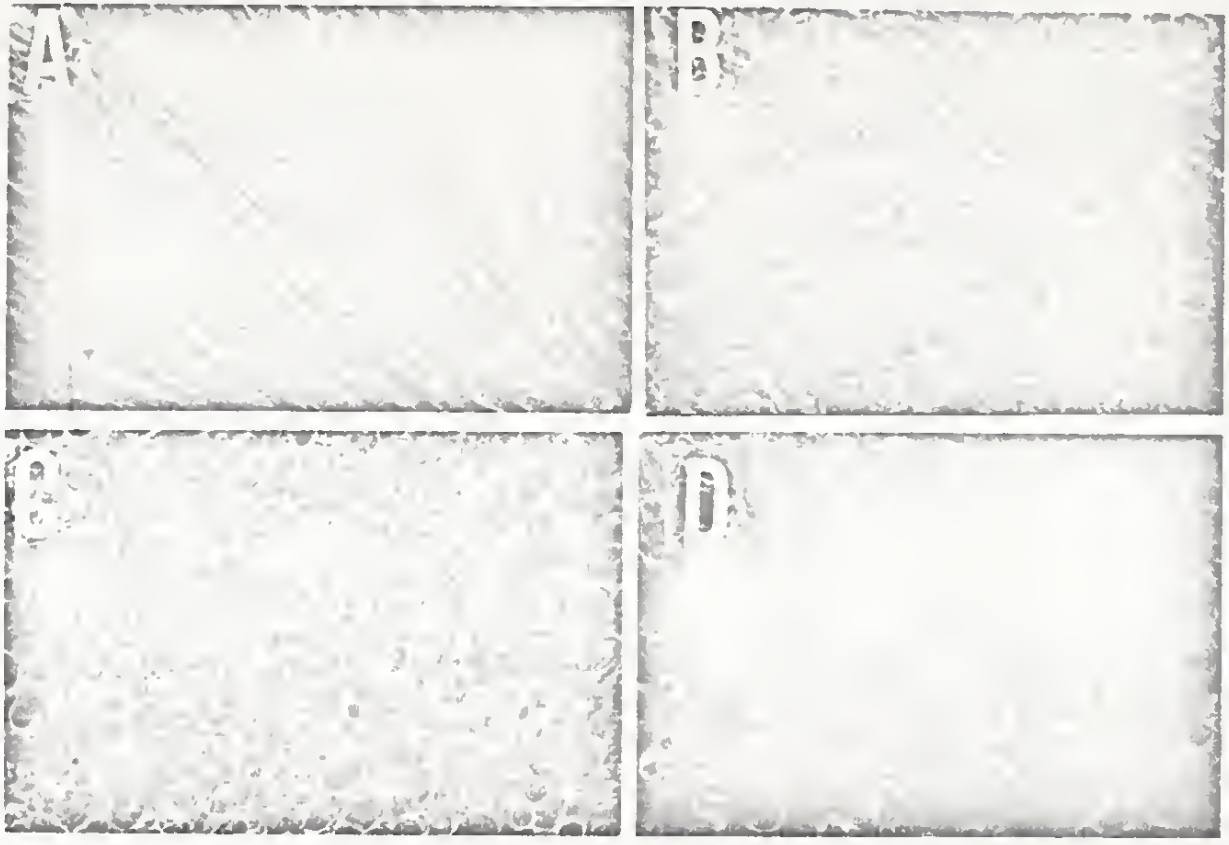


FIGURE 5. DOSE RESPONSE TO CADMIUM OF NORMAL CULTURED FIBROBLASTS

Cultured cells were incubated with media enriched with various quantities of cadmium for 48 hours at 37°C. Morphology of cells was observed under a phase contrast inverted microscope (Leitz) with magnification of 200X

A. medium with 0.1 µg cadmium/ml
B. medium with 0.3 µg cadmium/ml
C. medium with 0.7 µg cadmium/ml
D. medium with 1.1 µg cadmium/ml

From the results presented above, MKHD and normal cells apparently responded at approximately the same cytotoxic level of cadmium. One possibility is that MKHD cells excrete cadmium with equal efficiency as normals. This would explain the fact that both MKHD and normal cells had similar intracellular cadmium content when incubated in basal medium. At first glance these data may seem contradictory to the other experimental observations, namely, MKHD cells constantly had a higher intracellular cadmium content at medium cadmium concentrations above 0.1 µg/ml, and also death of MKHD cells occurred at a higher intracellular cadmium concentration than normal. Cadmium was reported to induce synthesis of metallothionein in various organisms. One role postulated for metallotheonein is detoxification of heavy metals by complexing with the heavy metal ions (95,100,104,115,116,139). The phenomenon of increased intracellular cadmium concentration at cell death and on incubation with cadmium containing medium in MKHD cultures may be explained by several postulations: metallothionein is more inducible; metallothionein has more metal-binding sites; or metallothionein in MKHD cells has a higher affinity for cadmium ions. All three possibilities would account for the fact that MKHD cells have excretory mechanisms of equal efficiency but higher intracellular concentrations of cadmium than normal cells.

Metal Uptake Experiment

To further clarify the mechanism of metal uptake in cultured fibroblasts, incorporation studies were done with both ^{64}Cu and ^{109}Cd.

Due to the short half-life of ^{64}Cu, a complete study of ^{64}Cu required several shipments of the radionuclide. To ensure identical conditions for all the experiments, similar radioactivity was added to all the cultures. Variation in the quantity of copper present in the medium was eliminated by addition of non-radioactive copper to make a uniform concentration of

9.7 μg/ml of culture medium. A high medium copper concentration was used with the idea that any difference in copper homeostasis between MKHD and normal cells would manifest itself more obviously under high copper conditions. The copper level used, however, was well below cytotoxic level to avoid any deviation caused by cell death. The results of incorporation studies of ^{64}Cu are presented in Table 3. The uptake rate of ^{64}Cu in the first hour was greater in MKHD cultures: about 3 times that of normal. Similar observations were reported recently (169). Except at the third hour, incorporation of ^{64}Cu was always greater in MKHD than in normal cells. The incorporation peak occurred at the third hour for normal cells and at the fifth hour for MKHD cells.

The study provides further evidence that incorporation or absorption of copper is not blocked in MKHD cells. The results agree with the finding of *in vivo* copper absorption studies in MKHD patients; namely, that uptake of copper from the intestinal content into the mucosal cell was probably normal (146,155,164,165,174). The higher accumulation of radioactivity in the MKHD cell may be due to either more efficient uptake or a less efficient excretory mechanism. Peaks occurring in uptake studies are probably the product of the combined effects of uptake and excretion. Presumably, cells will start to excrete a certain cation more efficiently when its intracellular level reaches a given limit. From the results presented, it can be seen that for normal cells, the peak occurred at the third hour with a value of 3,346 cpm/mg cell protein, while for MKHD cells, the peak occurred at the fifth hour at 8,894 cpm/mg cell protein. The tentative conclusion from this study may, thus, be that the excretory mechanism is less efficient in MKHD cell and requires a higher intracellular copper concentration to initiate it to work efficiently.

TABLE 3

COPPER-64 INCORPORATION STUDIES IN TISSUE CULTURE AT 37°C [a, b]

Time (hour)	1	3	5	7	10
MKHD cells (n=4)[c]	7.0±0.7	2.2±0.3	8.9±0.5	5.4±0.9	5.5±0.2
Normal cells (n=4)	2.5±0.7	3.3±0.7	2.2±0.5	2.0±0.2	2.4±0.6

a. incorporation is expressed as 10^{-3}x ^{64}Cu cpm/mg cell protein; values are means ± standard deviations

b. specific activity of ^{64}Cu is 910 µCi/mg

c. n denotes number of different cultures tested

Incorporation of copper is very likely to be affected by the presence of cadmium. The results of incorporation of ^{64}Cu in the presence of 0.09 µg/ml of cadmium are represented in Table 4. There was a general increase in uptake of copper in the presence of cadmium in both MKHD and normal cells. Due to inevitable variations in conditions under which the experiments were done, the simplest way to look at the effect of cadmium or copper uptake was to compare the ratio of MKHD to normal incorporated radioactivity in the presence and absence of cadmium. This is shown in Table 5. It is clear that cadmium facilitated the uptake of copper more in normal cells than in MKHD cells. This conclusion, however, may only be partially true. Cadmium can induce the synthesis of metallothionein in cultured fibroblasts. The major roles of metallothionein include detoxification of heavy metals as well as intracellular transport of copper. Assuming the correctness of the above two statements, presence of cadmium will stimulate increased copper uptake. If, as discussed previously, high intracellular copper concentration of MKHD cells when incubated with ^{64}Cu at 37°C was due to inefficient excretory mechanism, any metallothionein induced by cadmium would be involved in binding the excess intracellular copper first. Thus, the slight increase in copper incorporation in the presence of cadmium observed in MKHD cells may be the result of the masking effect of higher intracellular copper concentration due to an inefficient excretory mechanism. Another possibility is that the induced metallothionein in MKHD cells has a higher cadmium affinity than that in normal cells. Thus cadmium competes more favorably for binding in MKHD cells than in normal cells resulting in a smaller increase in ^{64}Cu incorporation in MKHD cells. Naturally, the observation can also be interpreted as a result of failure in induction of metallothionein by

TABLE 4

COPPER-64 INCORPORATION OF CULTURED FIBROBLASTS IN PRESENCE OF CADMIUM AT 37°C [a, b]

Time (hour)	1	3	5	7	10
MKHD cells (n=3)[c]	2.6±0.5	5.6±1.6	8.1±1.0	6.9±0.4	7.5±0.9
Normal cells (n=3)	2.2±0.4	6.4±0.8	8.6±2.9	6.0±0.7	7.1±0.5

a. incorporation is expressed as $10^{-3} \times {}^{64}Cu$ cpm/mg cell protein; values are means ± standard deviations

b. specific activity of ^{64}Cu is 910 μCi/mg; concentration of cadmium is 0.09 μg/ml

TABLE 5

EFFECT OF CADMIUM ON COPPER UPTAKE IN NORMAL AND MKHD CULTURED FIBROBLASTS COMPARED

Time (hour)		1	3	5	7	10
$\frac{\text{MKHD cells } {}^{64}\text{Cu cpm/mg protein}}{\text{Normal cells } {}^{64}\text{Cu cpm/mg protein}}$	at 37°C	2.8	0.7	4.1	2.6	2.3
$\frac{\text{MKHD cells } {}^{64}\text{Cu cpm/mg protein}}{\text{Normal cells } {}^{64}\text{Cu cpm/mg protein}}$	at 37°C presence of cadmium	1.2	0.8	0.9	1.1	1.1

cadmium in MKHD cells. The one general conclusion which can be deduced from the above two experiments is that the influx of copper is unaffected in MKHD cultured fibroblasts.

Uptake of cadmium was higher in MKHD than in normal cultures as shown in Table 6. Initial uptake rate of cadmium was about the same in both types of cultures. However, intracellular cadmium content of MKHD cells increased with increased time of incubation while normal cells always maintained the same intracellular cadmium level. From the results, it appeared that there was also a change in cadmium homeostasis in MKHD cells. The higher intracellular cadmium content of MKHD cells may be due to either an inefficient cadmium excretion mechanism or an increase in cadmium affinity of metallothionein. Presence of copper reduced uptake of cadmium. The data are represented in Table 7. This is expected since if metallothionein binds both cadmium and copper and is responsible for both detoxification and intracellular transport of metal ions, a mutation in metallothionein may affect both copper and cadmium binding. The decreased incorporation of radioactivity is probably the result of competition for binding of copper and cadmium to metallothionein. In the absence of copper, cadmium can bind to all available metal binding sites. The presence of copper will reduce the number of sites available for cadmium binding. This is true in both MKHD and normal cells. However, as shown in Tables 6 and 7, copper reduced cadmium binding more in MKHD than in normal cells.

To further investigate the relative change in copper and cadmium binding, a competitive uptake experiment was performed. The results are represented in Table 8. To compare the relative amount of radionuclides in the cells, the ratio ^{109}Cd cpm/mg cells protein to ^{64}Cu cpm/mg cell protein was computed. It can be seen that from the fifth hour onwards the

TABLE 6

CADMIUM-109 INCORPORATION STUDIES IN TISSUE CULTURE AT 37°C [a, b]

Time (hour)	1	3	5	7	10
MKHD cells (n=3)[c]	8.6±0.6	9.6±0.6	10.3±1.6	12.6±0.3	13.8±4.0
Normal cells (n=3)	7.7±0.5	7.4±0.3	5.9±0.0	7.4±0.3	7.1±0.6

a. incorporation is expressed as $10^{-4} \times {}^{109}Cd$ cpm/mg cell protein; values are means ± standard deviations

b. specific activity of ${}^{109}Cd$ is 713 μCi/μg

c. n denotes number of different cultures tested

TABLE 7

CADMIUM-109 INCORPORATION OF CULTURED FIBROBLASTS IN PRESENCE OF COPPER AT 37°C [a, b]

Time (hour)	1	3	5	7	10
MKHD cells (n=3)[c]	3.0±0.7	3.5±0.9	5.7±1.6	9.7±2.3	8.3±1.6
Normal cells (n=3)	1.0±0.2	2.2±0.9	8.4±1.9	12.0±3.6	12.1±1.4

a. incorporation is expressed as $10^{-4} \times {}^{109}Cd$ cpm/mg cell protein; values are means ± standard deviations

b. specific activity of ^{109}Cd is 713 μCi/μg; concentration of copper is 5 μg/ml

c. n denotes number of different cultures tested

TABLE 8

COMPETITIVE UPTAKE OF COPPER-64 AND CADMIUM-109 IN TISSUE CULTURE AT 37°C [a, b]

Time (hour)	1	3	5	7	10
MKHD cells (n=3)[c]	17.0±1.2	37.5±4.1	152.7±30.5	158.1±20.0	154.0±16.0
Normal cells (n=3)	18.2±5.0	36.2±7.6	50.8± 2.5	63.6±10.2	47.0± 5.0

a. values are means of $\frac{^{109}Cd \text{ cpm/mg cell protein}}{^{64}Cu \text{ cpm/mg cell protein}}$ ± standard deviations

b. specific activity of ^{64}Cu is 910 μCi/mg

specific activity of ^{109}Cd is 713 μCi/μg

c. n denotes number of different cultures tested

ratio was higher in MKHD than in normal cells. Since both cadmium and copper uptake in MKHD cultures were shown to be comparable to normal cultures, the difference in the ratio might be due to either an increase in the numerator, i.e. ^{109}Cd counts, or a decrease in the denominator, i.e. ^{64}Cu counts, or both. Increased intracellular cadmium concentration may be due to inefficient excretory mechanism, and due to an increase in cadmium binding sites or increased affinity of the cadmium binding ligand. The opposite is true for the observed decreased intracellular copper amount. This experiment does not provide enough evidence to point out which postulation is correct.

Metal Efflux Studies

Inefficient effluxes of copper and cadmium were demonstrated in MKHD cells in the previous experiments. To investigate this phenomenon, effluxes of ^{64}Cu and ^{109}Cd were studied by pulse labelling the cells with the radionuclides and then chased with non-radioactive medium as discussed in the Materials and Methods section. The idea behind the experiment was that any defect in the explusion of cations from the MKHD cells would result in a higher percent retention of radioactivity with respect to that at the end of the pulse labelling period.

Efflux of ^{64}Cu was studied at $37^{o}C$ and at $4^{o}C$. The results are presented in Table 9. At $37^{o}C$, except for the first and third hour of the chase, MKHD cells always retained more ^{64}Cu than normal cells. Since both the MKHD and normal cultures were exposed to non-radioactive basal media during the chase experiment, any difference in percent retention of radioactivity would indicate a change in the egress process of the cation. Thus, at $37^{o}C$, MKHD cells demonstrated an impaired efflux of copper. At $4^{o}C$, energy requiring processes would be retarded. Therefore, if the egress process involved an energy requiring step, lowering the temperature

TABLE 9

RETENTION OF COPPER-64 IN TISSUE CULTURE AT 37°C and 4°C [a, b]

Temp.	Cell Cultures	Time of Chase (hour) 0	1	3	5	7	10
37°C	MKHD cells (n=4)[e]	7346[c] ± 1726	23.4[d] ± 8.0	19.1 ± 5.5	30.3 ± 4.0	27.0 ± 5.4	20.9 ± 6.0
	Normal cells (n=4)	7670 ± 1430	25.7 ± 5.1	19.1 ± 2.7	17.8 ± 4.1	11.6 ± 2.5	12.2 ± 4.0
4°C	MKHD cells (n=5)		18.0 ± 5.5	22.5 ± 4.2	20.0 ± 4.0	26.7 ± 6.5	25.4 ± 5.1
	Normal cells (n=3)		11.4 ± 2.6	11.2 ± 1.8	12.7 ± 2.9	12.3 ± 0.2	15.7 ± 4.1

a. values are means ± standard deviations

b. specific activity of ^{64}Cu is 595 μCi/mg

c. units are ^{64}Cu cpm/mg cell protein

d. data at other hours of chase are percent of time zero counts

e. n denotes number of different cultures tested

would cause a slowing down of the egress of cation, resulting in an increase in retention of ^{64}Cu. From Table 9, it can be seen that lowering the temperature did not increase the percent retention except at the tenth hour. In fact, at $4^{o}C$, the percent retention in normal cells was lowered more than the MKHD cells, making the difference between the two more pronounced. Thus, apparently the efflux of copper from cultured fibroblasts is not an energy requiring process. Besides, impairment of copper efflux was also true at $4^{o}C$ in MKHD cells. Presence of cadmium in the chase medium was at first expected to decrease the percent retention of ^{64}Cu. However, as presented in Table 10, the opposite was observed. This is probably due to the induction effect of cadmium on metallothionein synthesis. Synthesis of more metallothionein during the chase period will help retaining more ^{64}Cu. Higher percent of ^{64}Cu was still retained by the MKHD cells in the presence of cadmium. The impairment of copper efflux in MKHD cells appears to be true at $37^{o}C$, $4^{o}C$ as well as in the presence of cadmium.

The efflux of cadmium from cultured fibroblasts was also studied at $37^{o}C$ and $4^{o}C$. The results are presented in Table 11. MKHD cultures tended to show a higher percent retention than normal at $37^{o}C$ and at $4^{o}C$. However, the difference between MKHD and normal cells was indistinguishable at both temperatures. The percent retention of ^{109}Cd also appeared not to vary with the change of temperature. From these observations one conclusion that can be reached is that, egress of cadmium from cultured fibroblasts is not an energy requiring process. Additionally, it is very probable that copper and cadmium effluxes involve different mechanism. Copper efflux mechanism is defective in MKHD cells. However, the present experimental data are not enough to say whether the cadmium efflux mechanism is defective or normal in MKHD cells.

TABLE 10

RETENTION OF COPPER-64 BY CULTURED FIBROBLASTS IN PRESENCE OF CADMIUM AT 37°C [a, b]

Time of chase (hour)	0	1	3	5	7	10
MKHD cells (n=5)[e]	4124[c] ± 468	34.2[d] ± 10.1	37.3 ± 8.5	37.2 ± 11.8	26.2 ± 6.4	37.2 ± 12.1
Normal cells (n=3)	2132 ± 378	12.0 ± 0.4	22.2 ± 1.2	18.8 ± 7.5	15.8 ± 5.2	25.1 ± 8.1

a. values are means ± standard deviations

b. specific activity of ^{64}Cu is 595 μCi/mg

c. units are ^{64}Cu cpm/mg protein

d. data at other time of chase are percent of time zero counts

e. n denotes number of different cultures tested

TABLE 11

RETENTION OF CADMIUM-109 IN TISSUE CULTURE AT 37°C AND 4°C [a, b]

Temp.	Cell Cultures	Time of Chase (hour) 0	1	3	5	7	10
37°C	MKHD cells (n=3)[e]	364950[c] ± 92040	90.2[d] ± 7.7	76.8 ± 9.7	77.8 ± 8.6	76.3 ± 13.1	56.1 ± 9.0
	Normal cells (n=3)	293408 ± 6167	93.0 ± 6.7	86.6 ± 1.0	65.8 ± 9.9	55.3 ± 8.2	43.6 ± 5.0
4°C	MKHD cells (n=3)		73.0 ± 16.5	79.3 ± 15.9	88.6 ± 5.0	48.3 ± 3.1	81.5 ± 5.6
	Normal cells (n=3)		76.5 ± 9.4	78.1 ± 17.0	56.0 ± 5.0	56.7 ± 6.2	65.9 ± 7.1

a. values are means ± standard deviations

b. specific activity of ^{109}Cd is 809 μCi/μg

c. units are ^{109}Cd cpm/mg cell protein

d. data at other hours of chase are percent of time zero counts

e. n denotes number of different cultures tested

In an attempt to investigate the relative affinity of copper and cadmium in MKHD cell, cultured fibroblasts were pulse labelled with ^{64}Cu or ^{109}Cd for 20 hours. The cultures were then chased with ^{109}Cd or ^{64}Cu, respectively. The ratio of ^{109}Cd radioactivity to ^{64}Cu radioactivity in the cell lysate was computed. After one hour of chase in the ^{64}Cu pulse, ^{109}Cd chase experiment the ^{109}Cd to ^{64}Cu radioactivity ratio was 154.7 ± 12.5 in MKHD and 52.4 ± 1.1 in normal cultures. The number of cell cultures tested in each case was 3. The higher ratio in MKHD may be due to a higher uptake or higher affinity for ^{109}Cd in MKHD cells. Results at later hours of the chase experiment were irregular and difficult to interpret, probably due to the involvement of a number of intricate events, e.g. induction of metallothionein by cadmium, efflux of copper, etc. ^{109}Cd pulse, ^{64}Cu chase experiment also yielded as a higher ^{109}Cd to ^{64}Cu radioactivity ratio (214.8 ± 2.2 in MKHD cells compared with 149.6 ± 1.4 in normal cells) at the first hour of the chase experiment. As discussed before, uptake of ^{64}Cu was faster in MKHD cells than normal in the first hour of incubation. Thus, the higher ^{109}Cd to ^{64}Cu ratio observed in this experiment cannot be due to a lower incorporation of ^{64}Cu in the MKHD cells. The other possibilities may then be a higher affinity for ^{109}Cd or an inability of copper to displace cadmium in MKHD cells.

The metal efflux studies as a whole indicate that the egress of copper and cadmium is not an active transport process. Copper efflux is impaired in MKHD cells. There is a possibility that cadmium efflux is also impaired. However, not enough evidence is presently available to support this supposition. The mechanism of copper and cadmium efflux is different. This will be discussed further in the general discussion. And, finally, MKHD cells seem to have a higher affinity for cadmium than normals.

Metal Induction of Metallothionein

According to results of the previous experiments, the primary defect of MKHD is in the efflux of copper from the cells. Since metallothionein is involved in intracellular transport of cations, any mutation in metallothionein can manifest itself as defective excretory mechanism. Thus, it is worthwhile to study the properties of metallothioneins in MKHD cells. Mutation of metallothionein may be in the structure or in the inducibility of the protein. These experiments were designed to investigate the inducibility of metallothionein in MKHD cells and in normals by copper and cadmium.

Metallothionein has a very high cysteine content. Thus, the induction of metallothionein was monitored by measuring the incorporation of L-(^{35}S) cysteine. Concurrence of ^{35}S and metal radioactivity peaks was used as an indication of metal induced metallothionein. Control experiments were run by incubating cultured fibroblasts with medium containing L-(^{35}S) cysteine only. The results are represented in Figure 6. Radioactivity incorporated in the first hour was (0.41 ± 0.06) x 10^5 cpm/mg cell protein in MKHD cells compared with (1.55 ± 0.05) x 10^5 cpm/mg protein in normal cells. The highest incorporated radioactivity in MKHD cells occurred at the tenth hour and was (3.50 ± 0.10) x 10^5 cpm/mg cell protein (4.0 nmole cysteine/mg cell protein). In normal cultures, the peak occurred at the seventh hour, with radioactivity of (5.0 ± 0.42) x 10^5 cpm/mg cell protein (5.70 nmole cysteine/mg cell protein). Thus, normal cells appeared to incorporate more cysteine than MKHD cells in the absence of exogenously added cations.

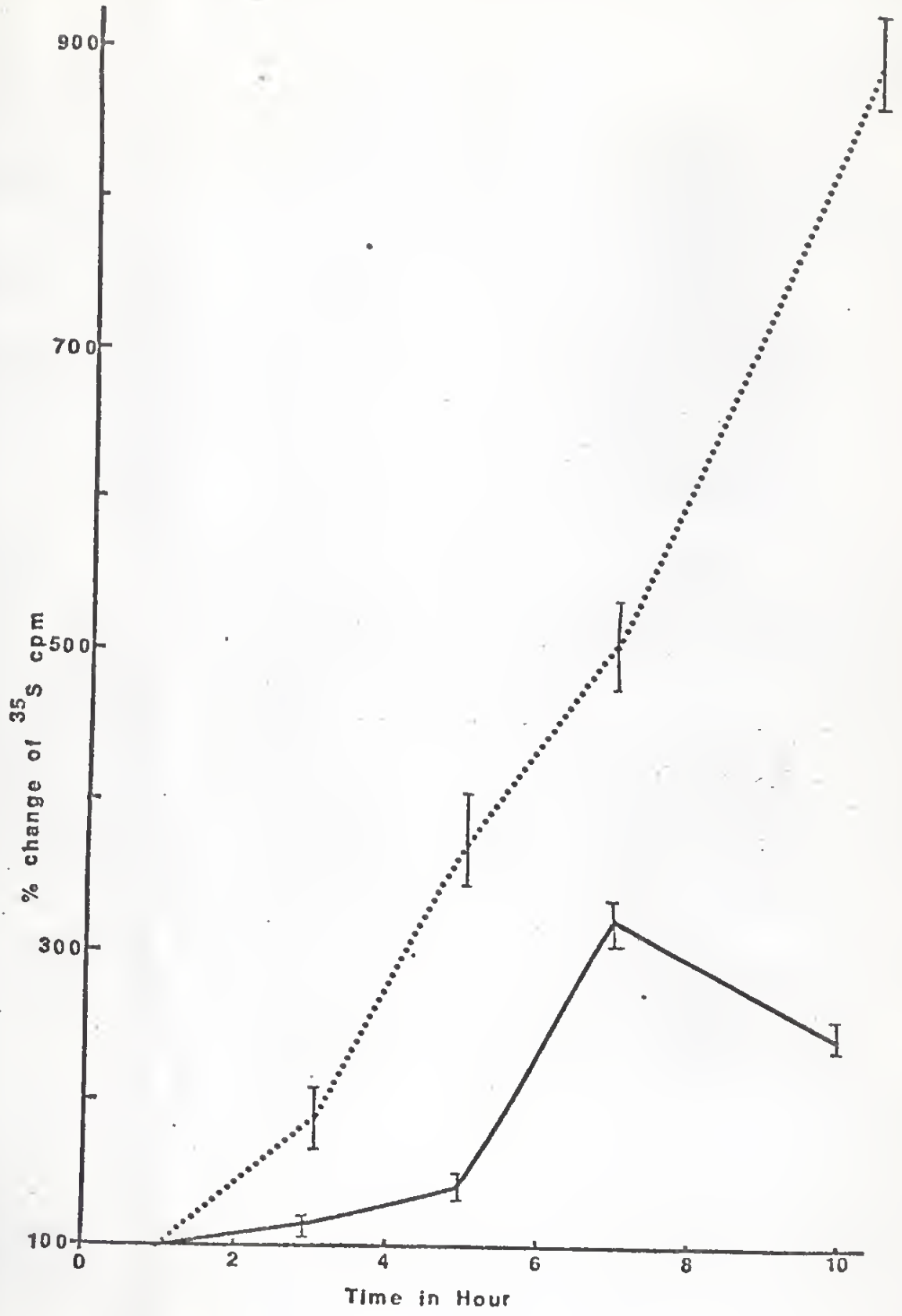
Inducibility of metallothionein by copper was examined by incubating the cultures with media containing both L-(^{35}S) cysteine and ^{64}Cu. The

FIGURE 6. INCORPORATION OF L-(^{35}S) CYSTEINE BY CULTURED FIBROBLASTS AT 37°C

Specific activity of L-(^{35}S) cysteine is 39.1 mCi/nmole. Radioactivity is expressed as cpm/mg cell protein. Results at various time periods are expressed as percent of the first hour incorporation. Values in the Figure are means of 2 different cultures of MKHD and normal fibroblasts. The first hour result for MKHD cells was (0.41 ± 0.06) x 10^5 cpm/mg cell protein and for normal cells was (1.5 ± 0.05) x 10^5 cpm/mg cell protein.

----------MKHD cells

——————— Normal cells


900
700
500
300
100
% change of ^{35}S cpm
0
2
4
6
8
10
Time in Hour

results are presented in Figure 7. Graph A is the induction profile for normal culture, and graph B is the induction profile for MKHD culture. From the graphs, it can be seen that incorporation of both ^{64}Cu and L-(^{35}S) cysteine peaked at the fifth hour in normal cells, while in MKHD cells ^{64}Cu incorporation peaked at the fifth hour and L-(^{35}S) cysteine incorporation peaked at the seventh hour. Comparing Figures 6 and 7, it is obvious that the presence of copper changed the incorporation pattern of cysteine. The incorporation peak of cysteine was shifted from hour 7 to hour 5 in normals, and from hour 10 to hour 7 in MKHD. Table 12 represents the amount of cysteine and copper corresponding to the mean incorporated radioactivity at each time period for both MKHD and normal cells. Comparing the amount of cysteine incorporated at the peak period in the presence and absence of copper (in normals: 4.08 nmole to 5.70 nmole; in MKHD: 3.57 nmole to 4.0 nmole) it is seen that cultured fibroblasts, both normals and MKHD, incorporated less cysteine in the presence of copper. From Table 12, it is also obvious that MKHD cells incorporated less cysteine than normal cells in the presence of copper.

Since the cysteine and copper radioactivity peaks coincided at the fifth hour in normal cells, it appeared that metallothionein was induced by copper. To prove whether or not metallothionein was really induced, the lysates of cells harvested at the fifth hour were applied to polyacrylamide gel electrophoresis. The gel columns after electrophoresis were sliced, dissolved, and counted for ^{35}S and ^{64}Cu. The electrophoretic profiles of normal and MKHD cells are shown in Figure 8. Graph A is for normal cells and graph B is for MKHD cells. In both graphs, no concurrent ^{35}S and ^{64}Cu peak are seen indicating that metallothionein is not induced by copper in MKHD or in normal cells.

FIGURE 7. INDUCED INCORPORATION OF L-(^{35}S) CYSTEINE IN CULTURED FIBROBLASTS BY COPPER-64 AT 37°C

Specific activity of L-(^{35}S) cysteine is 39.1 mCi/nmole and ^{64}Cu is 621 µCi/mg. Data were recorded as cpm/mg cell protein. Results at various time periods are expressed as percent of the first hour incorporation. Values in the Figure are means of 3 different cultures for both MKHD and normal fibroblasts.

———————^{35}S radioactivity

----------^{64}Cu radioactivity

A. normal cells
B. MKHD cells

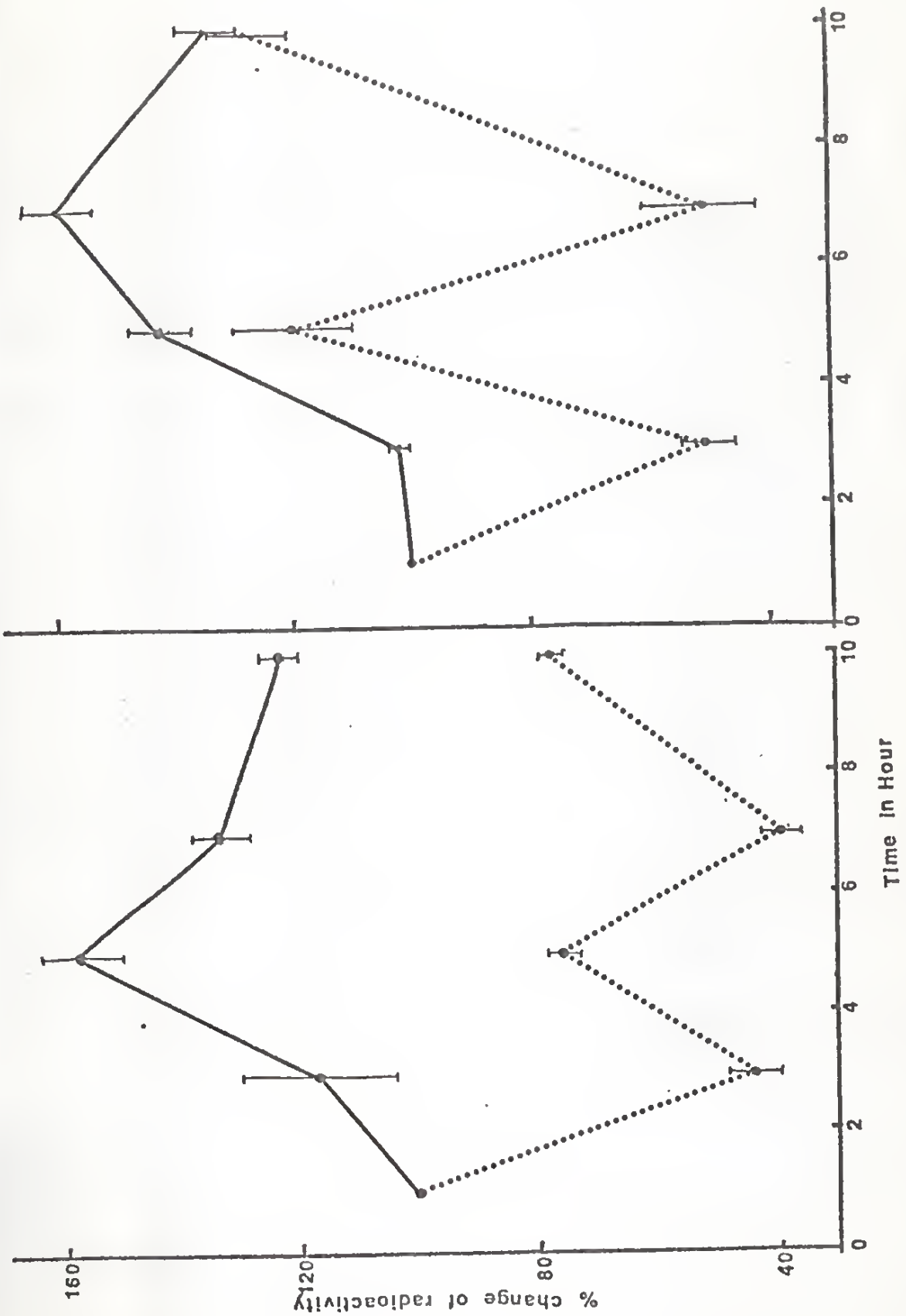

% change of radioactivity
160
120
80
40
0
2
4
6
8
10
Time in Hour

TABLE 12

COPPER INDUCED INCORPORATION OF CYSTEINE IN CULTURED FIBROBLASTS AT 37°C [a, b]

	Time (hour)									
	1		3		5		7		10	
	MKHD	NORMAL	MKHD	NORMAL	MKHD	NORMAL	MKHD	NORMAL	MKHD	NORMAL
L-(^{35}S) cysteine nmole/mg cell protein	2.21	2.62	1.69	2.97	3.20	4.08	3.57	3.41	2.67	2.92
^{64}Cu ng-atom/mg cell protein	0.52	0.60	0.21	0.29	0.62	0.50	0.26	0.25	0.67	0.50
nmole cysteine/ng atom ^{64}Cu	4.26	4.29	8.03	10.2	5.15	8.15	13.74	13.65	3.98	5.89

a. values are means of 3 different cultures for both MKHD and normal fibroblasts.

b. specific activity of L-(^{35}S) cysteine is 39.1 mCi/nmole

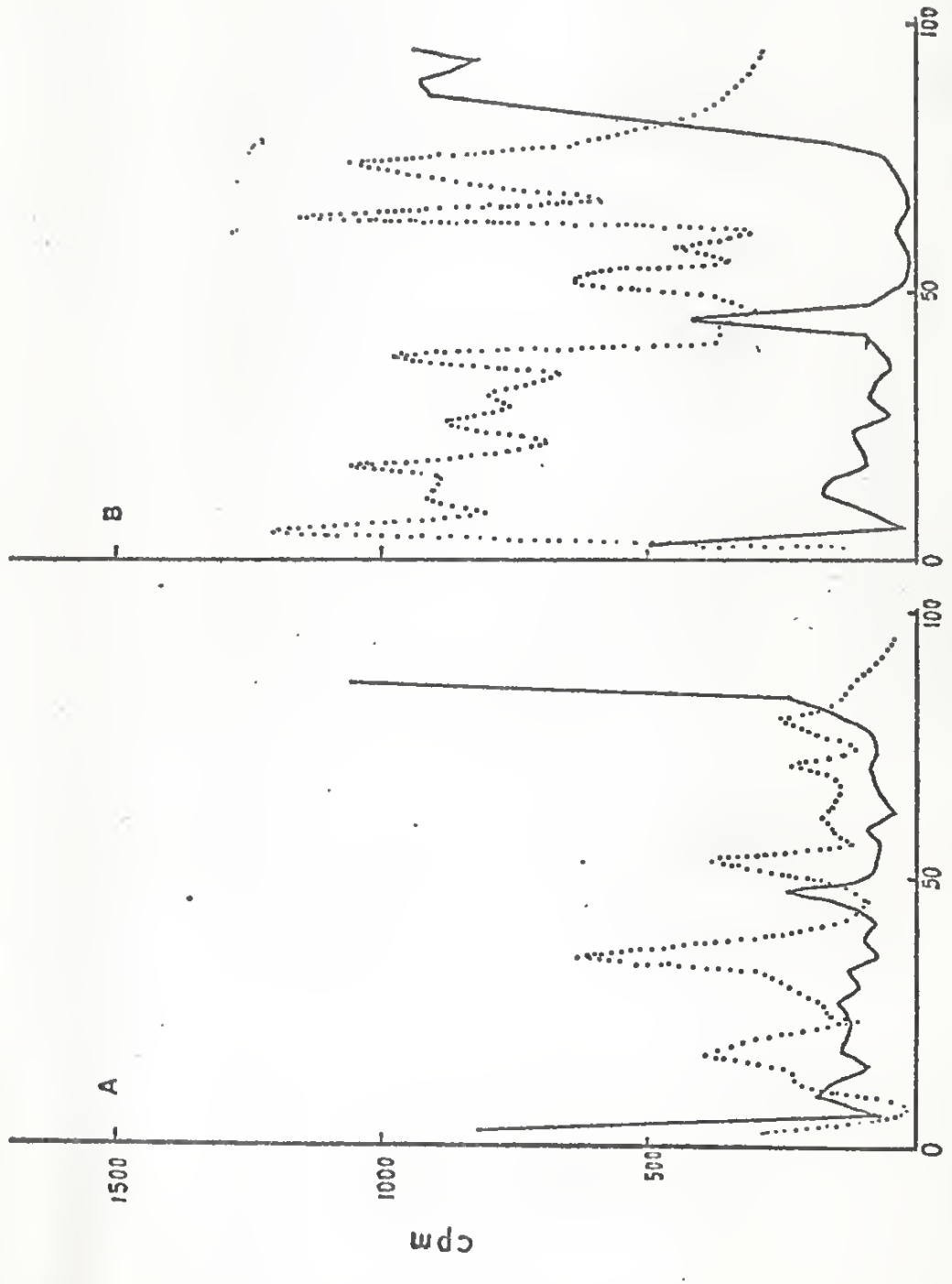
specific activity of ^{64}Cu is 621 mCi/mg

FIGURE 8. POLYACRYLAMIDE GEL ELECTROPHORESIS PATTERNS OF LYSATE OF CELLS INCUBATED WITH L-(^{35}S) CYSTEINE AND COPPER-64 FOR 5 HOURS AT 37°C

Distance on the gel was measured from top of the gel column ----------^{64}Cu radioactivity

_____^{35}S radioactivity

A. normal cells
B. MKHD cells


A
B
1500
1000
500
cpm
0
50
100
Distance in mm

Cadmium was shown to be able to induce metallothionein biosynthesis in intact animals as well as in certain types of cell. Thus, it is interesting to examine the effect of cadmium on cultured fibroblasts of MKHD patients. Both normal and MKHD cultures were incubated with L-(^{35}S) cysteine and ^{109}Cd at 37°C. Cells harvested at time intervals were analyzed for ^{35}S and ^{109}Cd content. The results are represented in Figure 9. Graph A represents the result for normal culture. A ^{109}Cd peak occurred at the third hour. However, cysteine incorporation appeared to peak at the seventh hour, and plateaued at this level till the tenth hour. Graph B represents the result for MKHD culture. In MKHD cells, ^{109}Cd incorporation also peaked at the third hour. A small ^{109}Cd peak appeared to start at the tenth hour. Cysteine incorporation profile indicated two peaks: one at the third hour and one at the tenth hour. From Figures 6 and 9 it can be seen that the incorporation profile of cysteine was very similar in the presence and absence of cadmium for both normal and MKHD cells. However, when comparing the amount of cysteine incorporated into cells, it is obvious that the presence of cadmium greatly enhanced the uptake of cysteine in both normal and MKHD cultures. This is shown in Table 13. The quantity of cysteine and cadmium shown in the Table was computed from the mean radioactivity of the corresponding radionuclide at the same time interval. The highest amount of cysteine incorporated by normal cultures in the presence of cadmium was 9.48 nmole/mg cell protein compared with 5.70 nmole/mg cell protein in the absence of cadmium. MKHD cells also incorporated much more cysteine in the presence of cadmium (9.0 nmole/mg cell protein) compared with 4.0 nmole/mg cell protein in the absence of cadmium.

To determine whether metallothionein was induced by cadmium in the cultured fibroblasts, lysates of cells harvested at time intervals

FIGURE 9. INDUCED INCORPORATION OF L-(^{35}S) CYSTEINE IN CULTURED FIBROBLASTS BY CADMIUM-109 AT 37°C

Specific activity of L-(^{35}S) Cysteine is 39.1 mCi/nmole and ^{109}Cd is 727 μCi/mg. Data were recorded as cpm/mg cell protein. Results at various time intervals were expressed as percent of the first hour incorporation. Values in the Figure are means of 3 different cultures for both MKHD and normal fibroblasts.

———————^{35}S radioactivity

----------^{109}Cd radioactivity

A. normal cells
B. MKHD cells

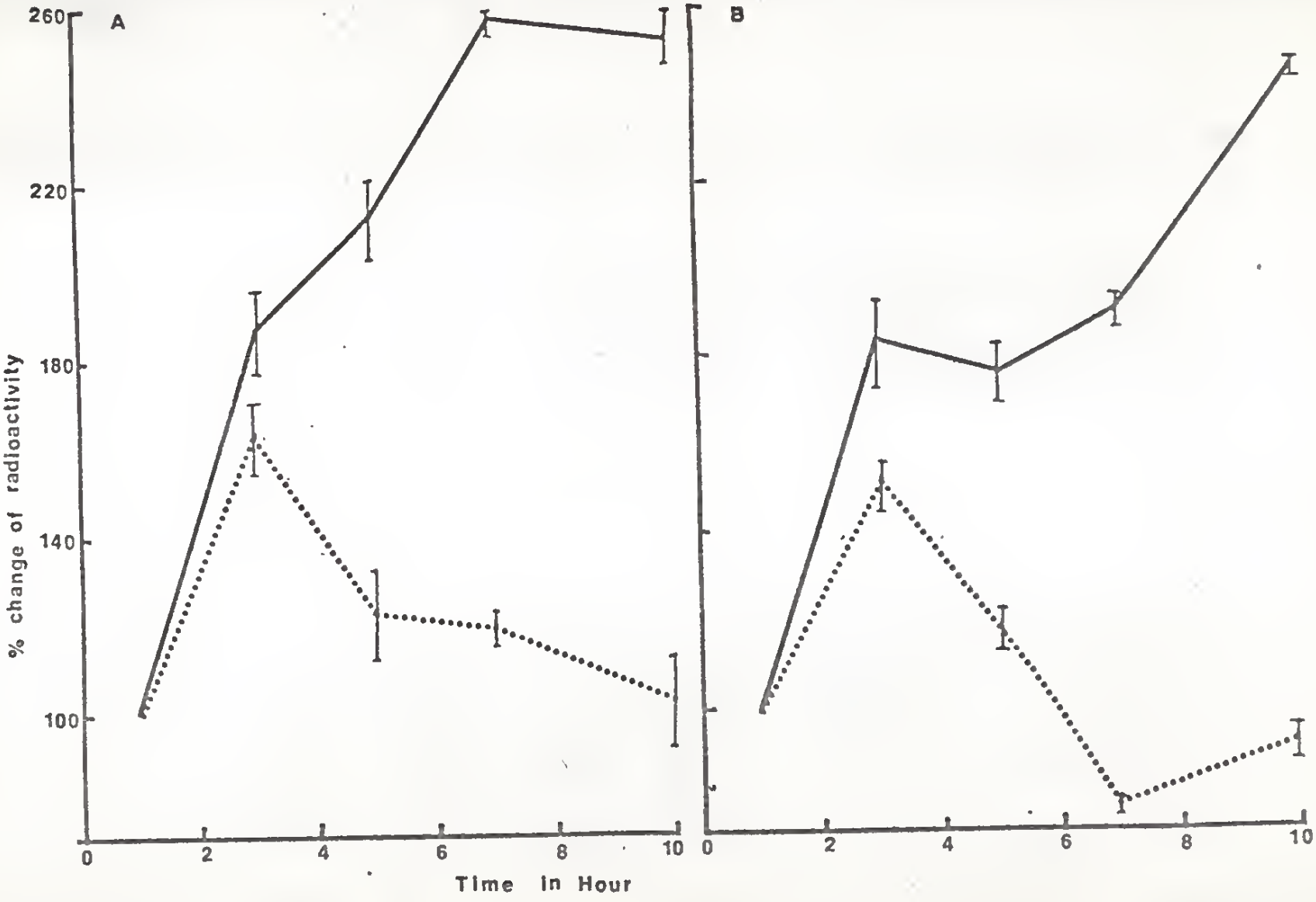

A
B
260
220
180
140
100
% change of radioactivity
0
2
4
6
8
10
Time In Hour

TABLE 13

CADMIUM INDUCED INCORPORATION OF CYSTEINE IN CULTURED FIBROBLASTS AT 37°C [a, b]

	Time (hour)									
	1		3		5		7		10	
	MKHD	NORMAL	MKHD	NORMAL	MKHD	NORMAL	MKHD	NORMAL	MKHD	NORMAL
L-(^{35}S) cysteine nmole/mg cell protein	3.93	3.59	7.69	6.81	7.85	7.82	7.52	9.48	9.02	9.31
^{109}Cd pg-atom/mg cell protein	0.95	0.60	1.35	1.10	1.05	0.80	0.70	0.70	0.90	0.60
nmole cysteine/pg-atom ^{109}Cd	4.14	5.98	5.70	6.20	7.48	9.78	10.74	13.54	10.02	15.52

a. values are means of 3 different cultures for both MKHD and normal fibroblasts

b. specific activity of L-(^{35}S) cysteine is 39.1 mCi/nmole

specific activity of ^{109}Cd is 727 μCi/μg

corresponding to ^{35}S peaks were applied to polyacrylamide gel electrophoresis. The electrophoretic patterns were constructed as before after slicing the gel columns and counted for ^{35}S and ^{109}Cd. Figure 10 presents the electrophoretic profiles at the third hour with A for normal and B for MKHD cells. Figure 11 presents the electrophoretic profiles at the tenth hour with A for normal and B for MKHD cells. Single ^{109}Cd peaks were observed in graphsA and B of Figure 10. The ^{109}Cd peak in graph A coincided with a small ^{35}S peak near the origin (top) of the gel column, while that in graph B coincided with a big ^{35}S peak near the end of the gel column. Since there was a large difference in mobility of the 2 ^{109}Cd peaks, they probably represent 2 different cadmium binding peptides. In Figure 11 A, though two ^{109}Cd peaks were present, only one coincided with a ^{35}S peak. A single ^{109}Cd peak coincident with a ^{35}S peak was also present in graph B of Figure 11. The concurrent ^{109}Cd and ^{35}S peaks in A and B appeared to have nearly identical mobilities indicating approximately equal molecular weight for the cadmium binding peptides. As proposed previously, concurrent metal and cysteine radioactivity peaks indicate the presence of metallothionein. Figure 11 demonstrats the induction of metallothionein by cadmium in both normal and MKHD cells. Due to a slightly higher mobility of the ^{109}Cd peak in B, metallothionein in MKHD cells appeared to have a smaller molecular weight than that in normal cells. There are controversies on the time course of cadmium induced metallothionein synthesis. All the observations reported in the literature were made on livers of intact rats. No in vitro experiment has been reported. Onset of metallothionein synthesis in vivo has been reported to be at 5 hours (204), 6 hours (112), 3 to 4 hours (205), between 5 and 24 hours (117) or at 24 hours (120) after administration of cadmium. These discrepancies

FIGURE 10. POLYACRYLAMIDE GEL ELECTROPHORESIS PATTERNS OF LYSATE OF CELLS INCUBATED WITH L-(^{35}S) CYSTEINE AND CADMIUM-109 FOR 3 HOURS AT 37°C

Distance on the gel was measured from top of the gel column ----------^{109}Cd radioactivity

_____^{35}S radioactivity

A. normal cells
B. MKHD cells

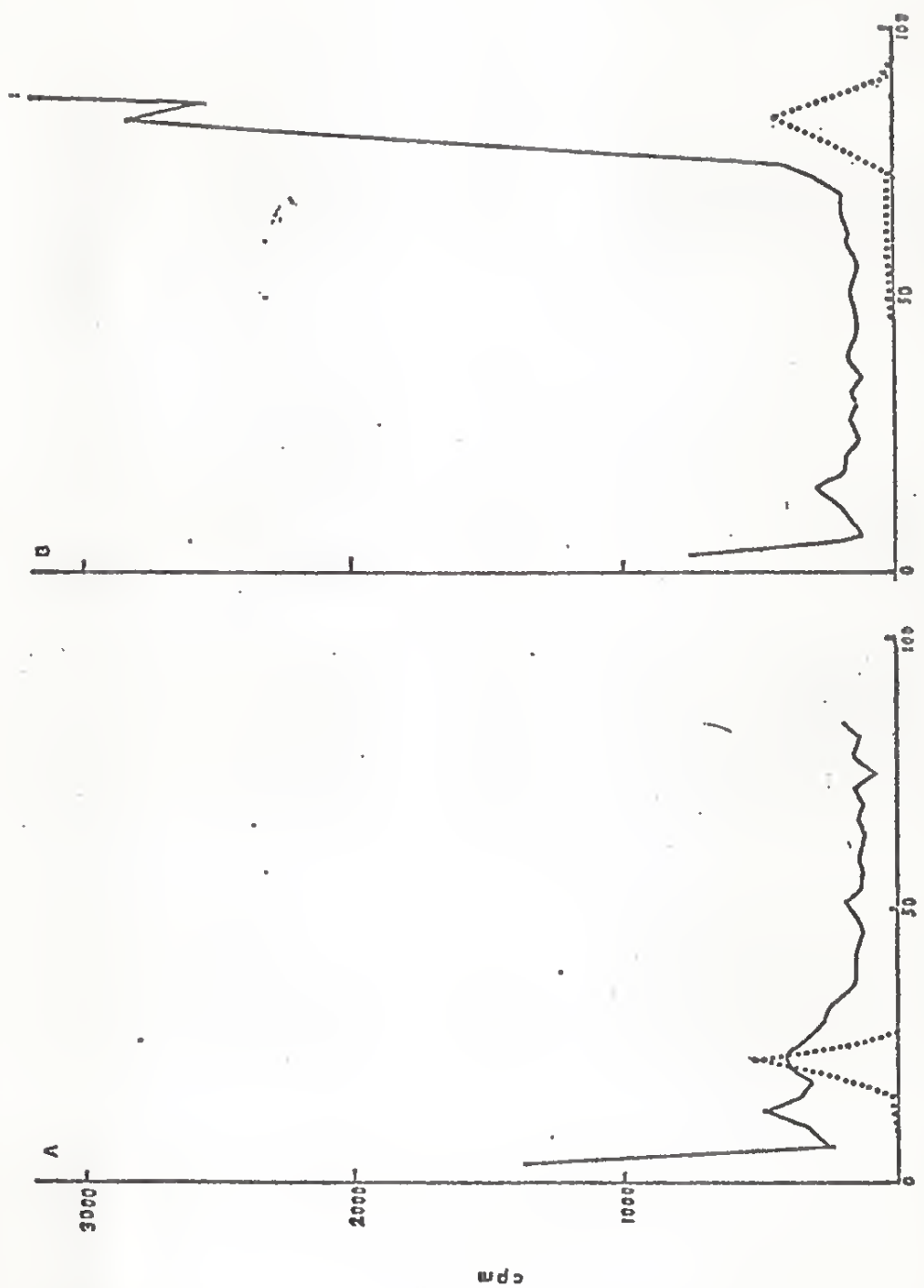
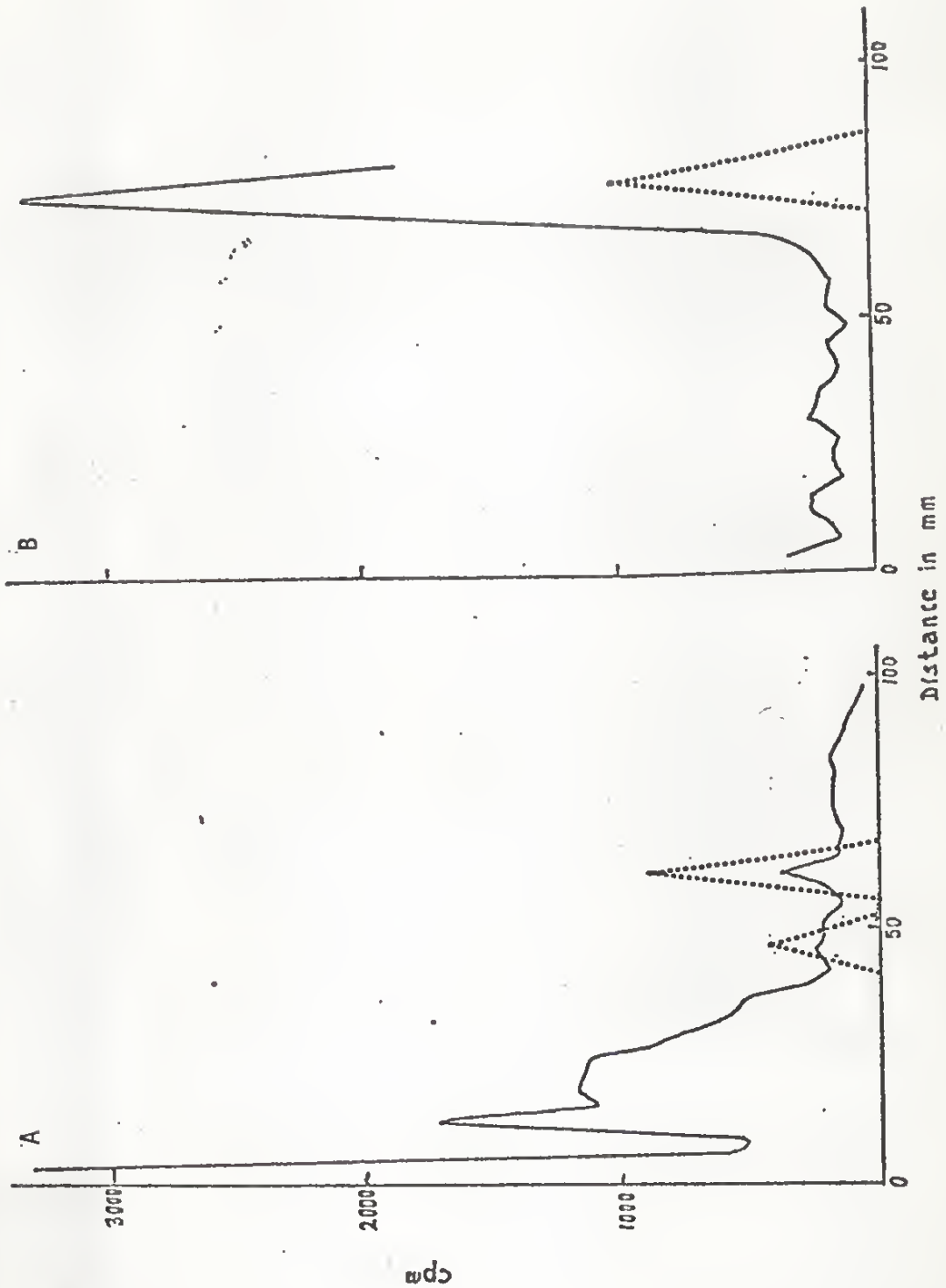

A
B
3000
2000
1000
cpm
0
50
100
Distance in mm

FIGURE 11. POLYACRYLAMIDE GEL ELECTROPHORESIS PATTERNS OF LYSATE OF CELLS INCUBATED WITH L-(^{35}S) CYSTEINE AND CADMIUM-109 FOR 10 HOURS AT 37°C

Distance on the gel was measured from top of the gel column----------^{109}Cd radioactivity

_____^{35}S radioactivity

A. normal cells
B. MKHD cells


A
B
cpm
3000
2000
1000
0
50
100
Distance in mm

may be due to different methods of administering the cation as well as the criteria used to indicate onset of metallothionein synthesis. The in vitro experiment reported here indicated that in human cultured fibroblasts the synthesis of metallothionein might have begun before the tenth hour after administering the cation. This result, though not similar to any of those reported, indicates that cells in culture have the same kind of response to cadmium as the intact animal (112,117,120,204,205).

The overall result of the metal induction experiments indicated that induction of metallothionein is not defective in MKHD cells. However, the metallothionein induced by cadmium in MKHD cells appeared to be smaller than that in normal cells. Copper is unable to induce metallothionein synthesis in either MKHD or normal cultured fibroblasts.

Preparation and Characterization of Metallothionein

Metallothionein was isolated from post-mortem human livers. All the livers were stored frozen at -37°C. Before the isolation procedure, they were thawed at 4°C overnight. MKHD patient liver was macroscopically indistinguishable from normal human liver. Ethanol fractionation of homogenized liver and lyophilization gave a yellowish solid. Quantities of the lyophilized solid varied between different extractions, but on an average about 1 % of the wet weight of liver was recovered. This extract had high zinc (71 mg-atom/g protein), and relatively low copper (16.3 mg-atom/g protein) and cadmium (77 µg-atom/g protein) content.

Figure 12 represents a typical Sephadex G-75 gel filtration pattern of the lyophilized material. The peak marked MT represents the metallothionein fractions. The last peak which has an intense 250 nm aborbance and high cadmium content was at first mistaken to be the metallothionein peak. The properties of the two peaks are compared in Table 14. Further

FIGURE 12. SEPHADEX G-75 CHROMATOGRAPHY OF LYOPHILIZED HUMAN LIVER EXTRACT IN 0.02 M TRIS-0.005 M HCl BUFFER, pH 8.6

Flow rate was 30 ml/hour, and volume of fractions were 20 ml. Peak marked MT represented metallothionein fractions.

----------absorbance of cadmium measured by atomic absorption spectrophotometer

————————250 nm absorbance

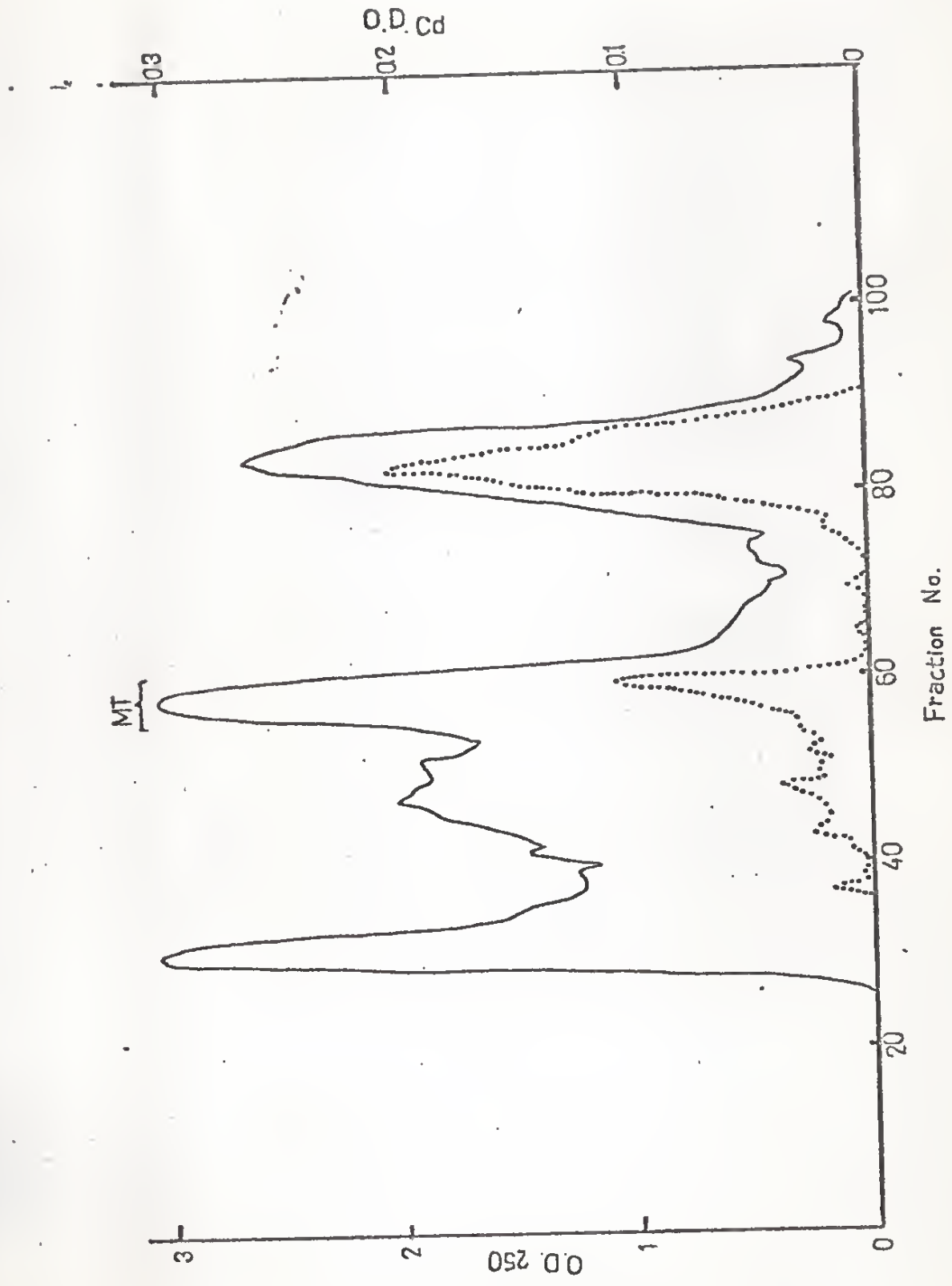

O.D. Cd
0.3
0.2
0.1
0
MT
O.D. 250
3
2
1
0
20
40
60
80
100
Fraction No.

TABLE 14

PROPERTIES OF THE SEPHADEX G-75 CHROMATOGRAPHY PEAKS OF LYOPHILIZED LIVER HOMOGENATE

	"MT" Peak	"Last" Peak
% protein recovery from lyophilized extract	3.35	1.81
total metal (Cu, Cd, Zn) µg-atom/g protein	418	52
nmole sulfhydryl/g protein	2.280	0.042

analysis on the last peak showed that it contained only some low molecular weight peptides. The MT peak eluted from the column had a yellowish color. Upon standing at 4°C for more than 24 hours, a white precipitate would settle out. The white precipitate was insoluble even in large volumes of buffer. On application of the MT fraction to the DEAE Sephadex A-25 column, the yellow color would stay on top of the gel.

The MT fraction was separated into 2 fractions by a DEAE Sephadex A-25 column eluted with a linear gradient with 0.02 M Tris-0.005 M HCl, pH 8.6 to 0.2 M Tris-0.05 M HCl, pH 8.6. The elution chromatogram is shown in Figure 13. The gradient started at fraction 60 and ended at fraction 174. The peak eluted before the end of the gradient was designated as metallothionein-1 (MT-1) while the one that came after the end of the gradient was designated as metallothionein-2 (MT-2). Both MT-1 and MT-2 appeared as clear colorless fractions. Isolation and purification of metallothionein from MKHD liver was stopped at this stage. The properties of normal MT-1, MT-2 and MKHD MT-1, MT-2 are compared in Table 15. To check the purity of the MT-1 and MT-2 obtained from the DEAE Sephadex A-25 column, 100 µg of the proteins were analyzed by electrophoresis. The gels were stained and scanned at 550 nm. The scanned gel pattern of MT-1 is shown in Figure 14, and that of MT-2 in Figure 15. The distance along the gel was measured from the top of the gel column. From the Figures it can be seen that the partially purified proteins contained only 1 or 2 major components.

Normal MT-1 was further purified by passing through a DEAE Sephadex A-25 column equilibrated with 0.03 M Tris-0.0075 M HCl, pH 8.6 buffer. The pure protein came out as sharp peak as shown in Figure 16. MT-2 was purified on a DEAE Sephadex A-25 column equilibrated with 0.03 M Tris-0.0075 M HCl, pH 8.6 and eulted with 0.06 M Tris-0.015 M HCl, pH 8.6. The chromatogram is depicted in Figure 17.

FIGURE 13. DEAE SEPHADEX A-25 CHROMATOGRAPHY OF MT FRACTION

Column was washed first with 600 ml of 0.02 M Tris-0.005 M HCl pH 8.6, followed by 1,600 ml of gradient, and then 600 ml of limiting buffer (0.2M Tris-0.05 M HCl, pH 8.6). Fractions of 14 ml were collected.

----------absorbance of cadmium measured by atomic absorption spectrophotometer

————————250 nm absorbance

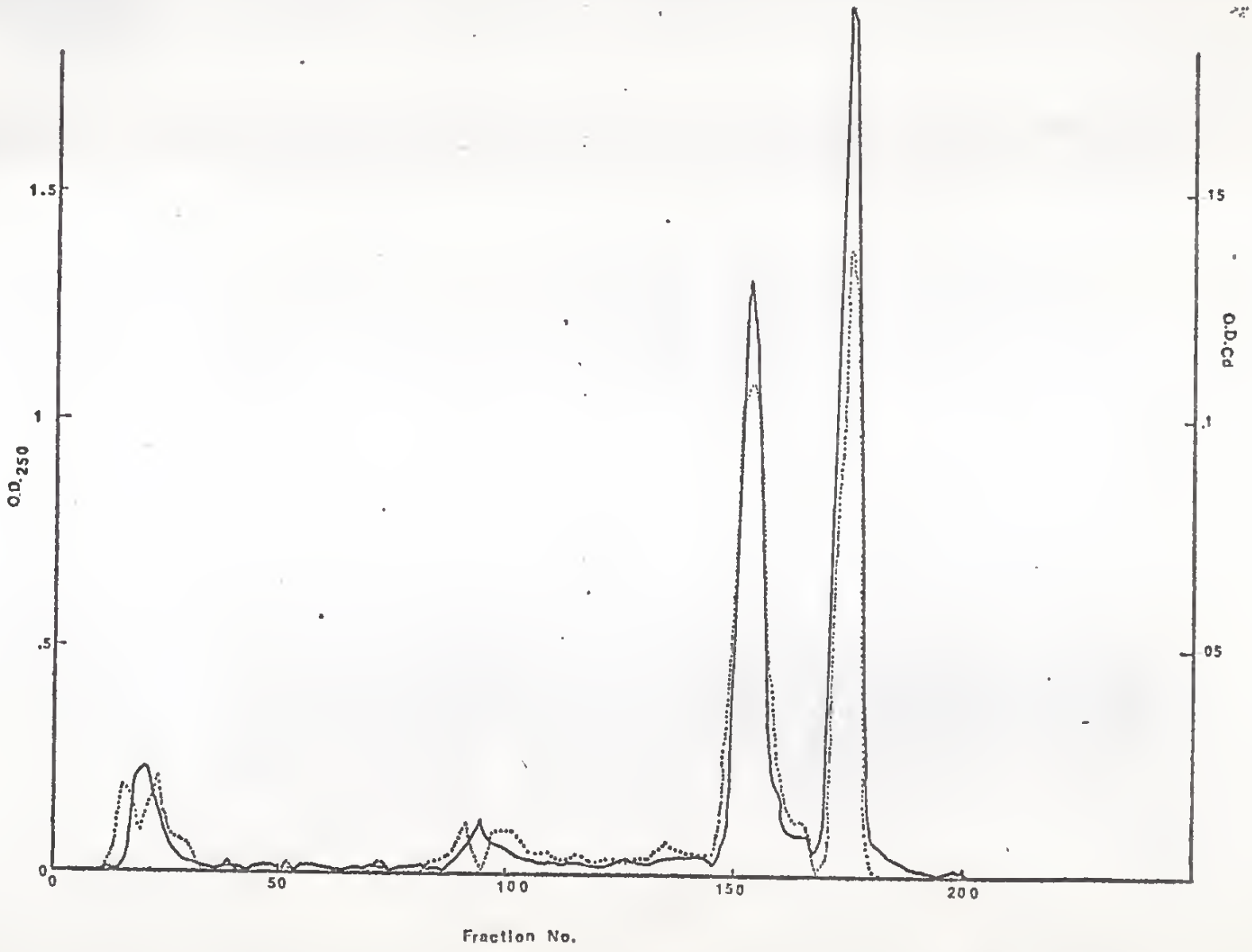

1.5
1
.5
0
O.D.250
15
1
.1
05
O.D.Cd
0
50
100
150
200
Fraction No.

TABLE 15

PROPERTIES OF PARTIALLY PURIFIED NORMAL AND MKHD HEPATIC METALLOTHIONEIN

Partially purified metallothionein	Sulfhydryl nmole / mg protein	Copper ng-atom / mg protein	Cadmium pg-atom / mg protein	Zinc ng-atom / mg protein	Total Metal ng-atom / mg protein
Normal:					
MT_1	3.16	12.78	75.0	16.20	29.00
MT_2	2.55	5.89	238.0	17.70	23.60
MKHD					
MT_1	1.96	2.49	56.1	1.30	3.80
MT_2	1.44	0.93	50.4	3.40	4.38

FIGURE 14. POLYACRYLAMIDE GEL ELECTROPHORESIS SCANNED GEL PATTERN OF PARTIALLY PURIFIED MT-1

Distance was measured from top of the gel
Gel was scanned at 550 nm

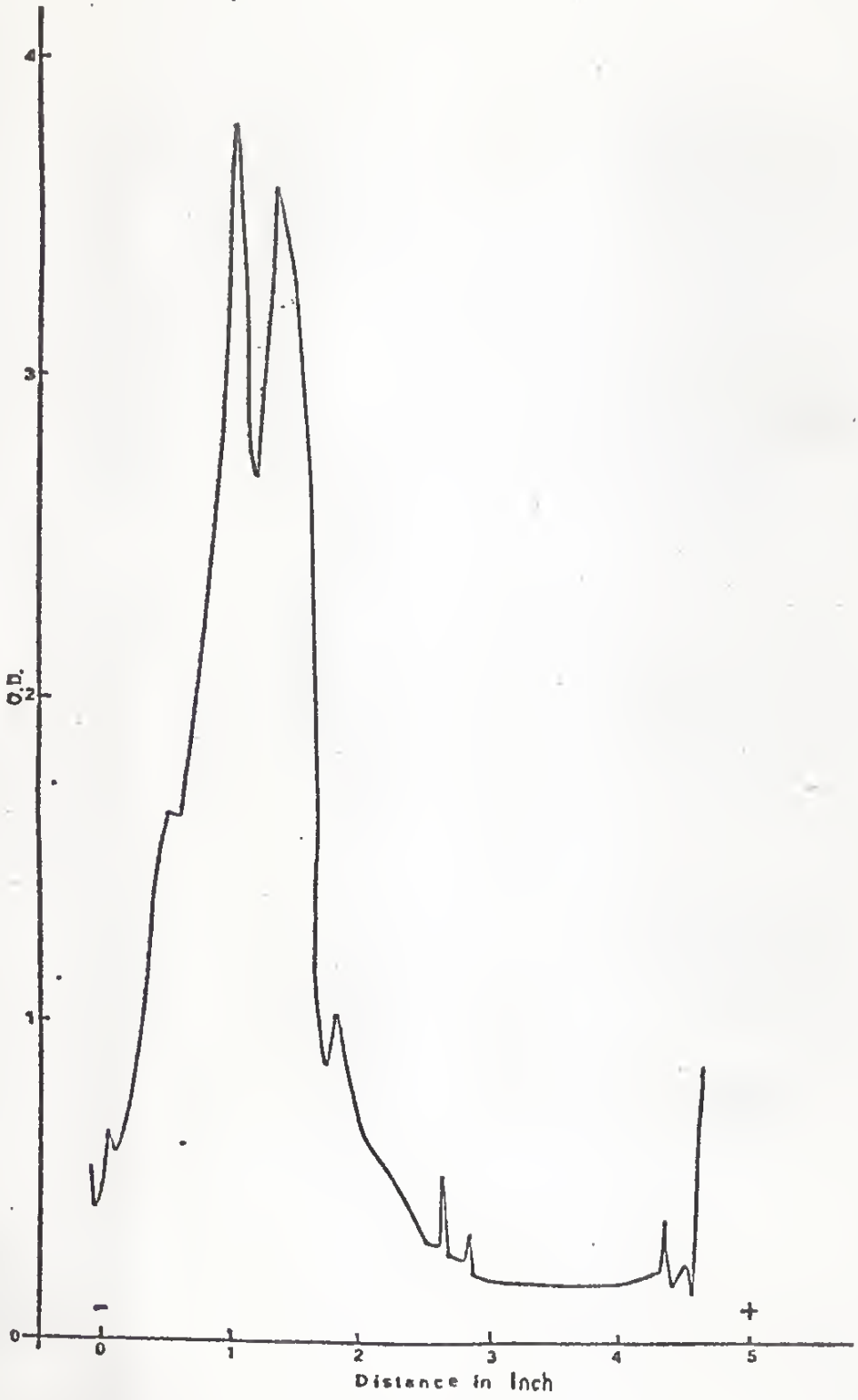

O.D.
4
3
2
1
0
0
1
2
3
4
5
-
+
Distance in Inch

FIGURE 15. POLYACRYLAMIDE GEL ELECTROPHORESIS SCANNED GEL PATTERN OF PARTIALLY PURIFIED MT-2

Distance was measured from top of the gel
Gel was scanned at 550 nm

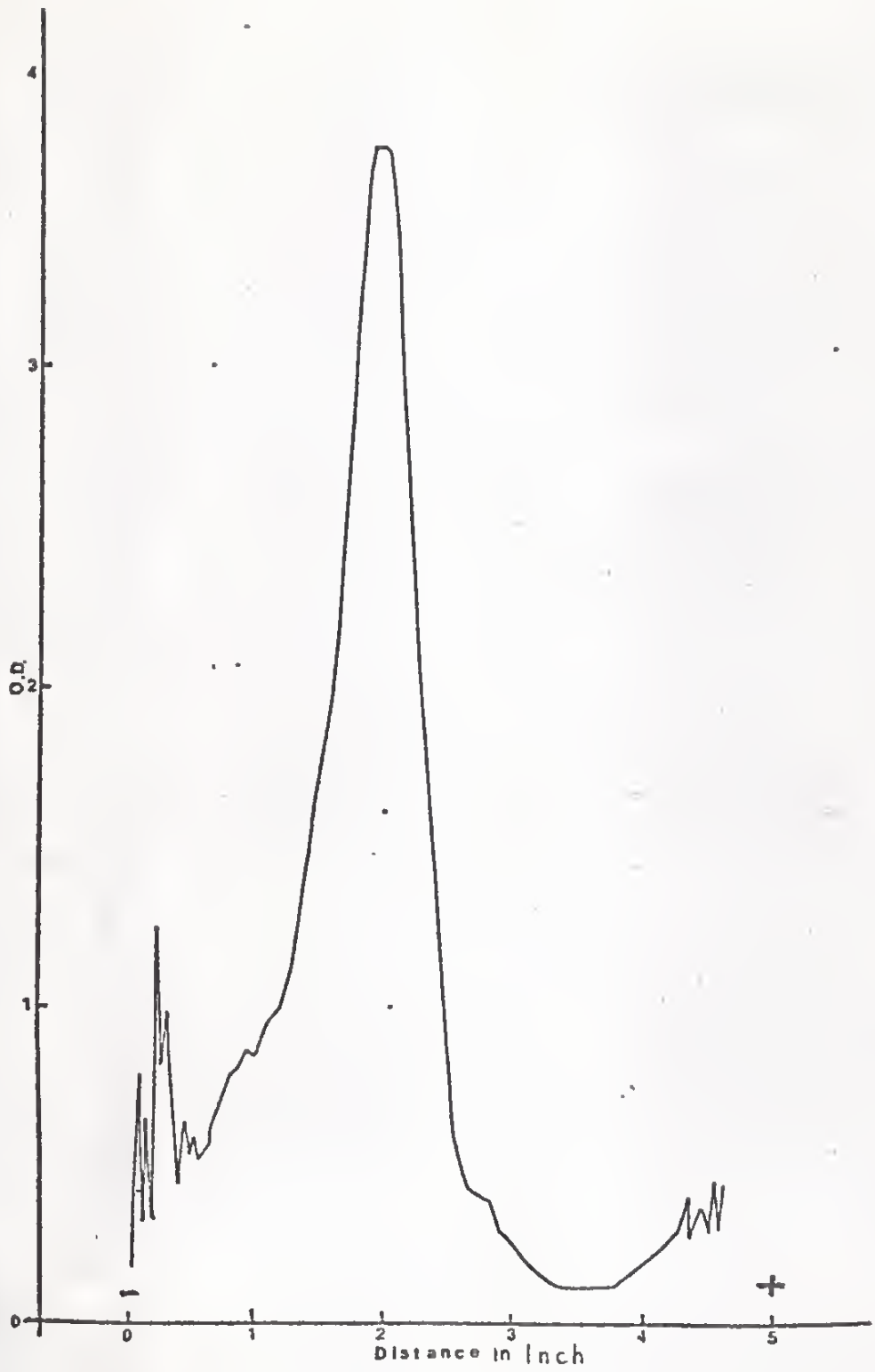

4
3
2
1
0
O.D.
-
+
0
1
2
3
4
5
Distance in Inch

FIGURE 16. DEAE SEPHADEX A-25 CHROMATOGRAPHY OF PARTIALLY PURIFIED MT-1

Buffer used to elute MT-1 was 0.03 M Tris-0.0075 M HCl pH 8.6. Ten ml fractions were collected.

----------absorbance of cadmium measured by atomic absorption spectrophotometer

————————250 nm absorbance

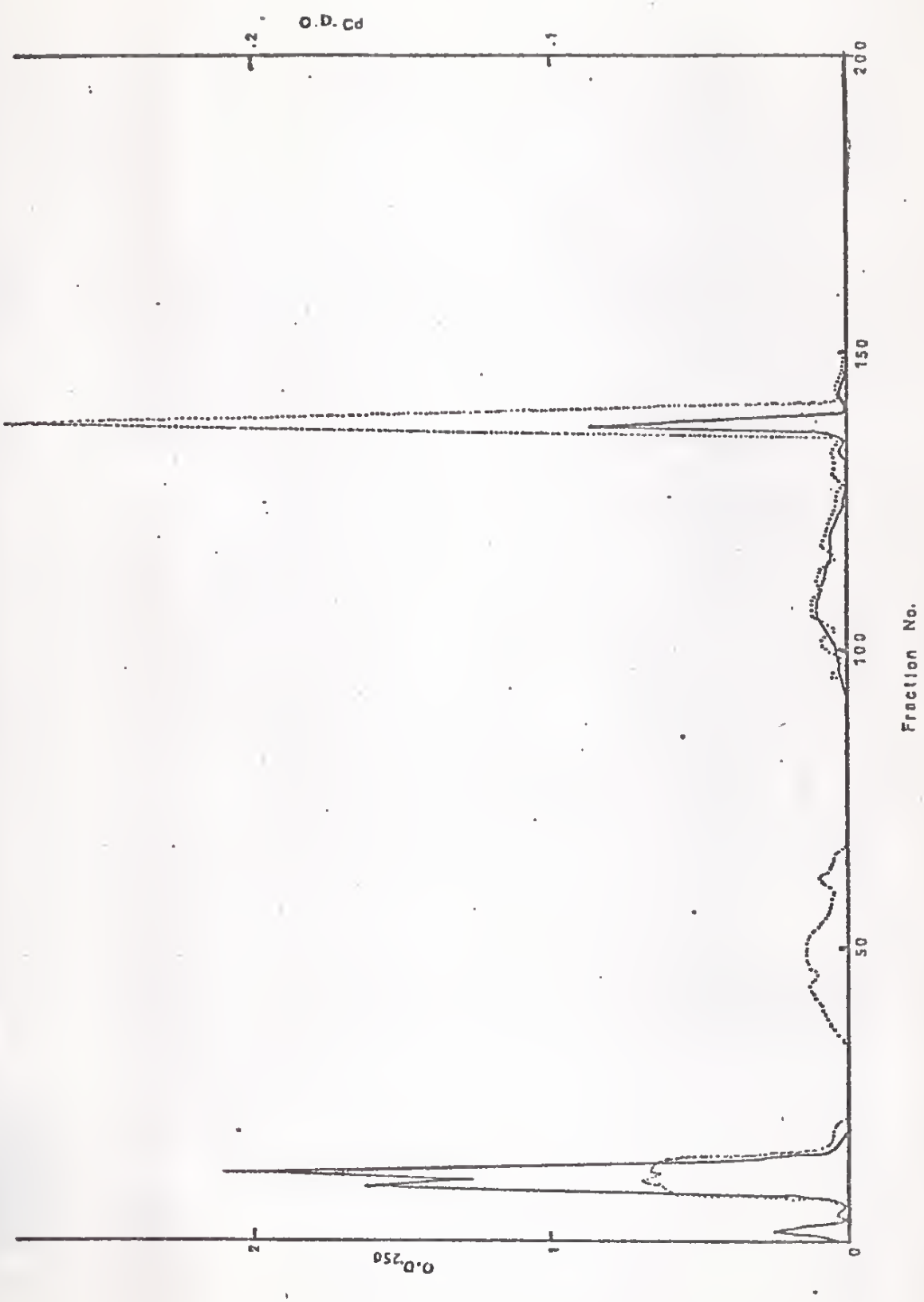
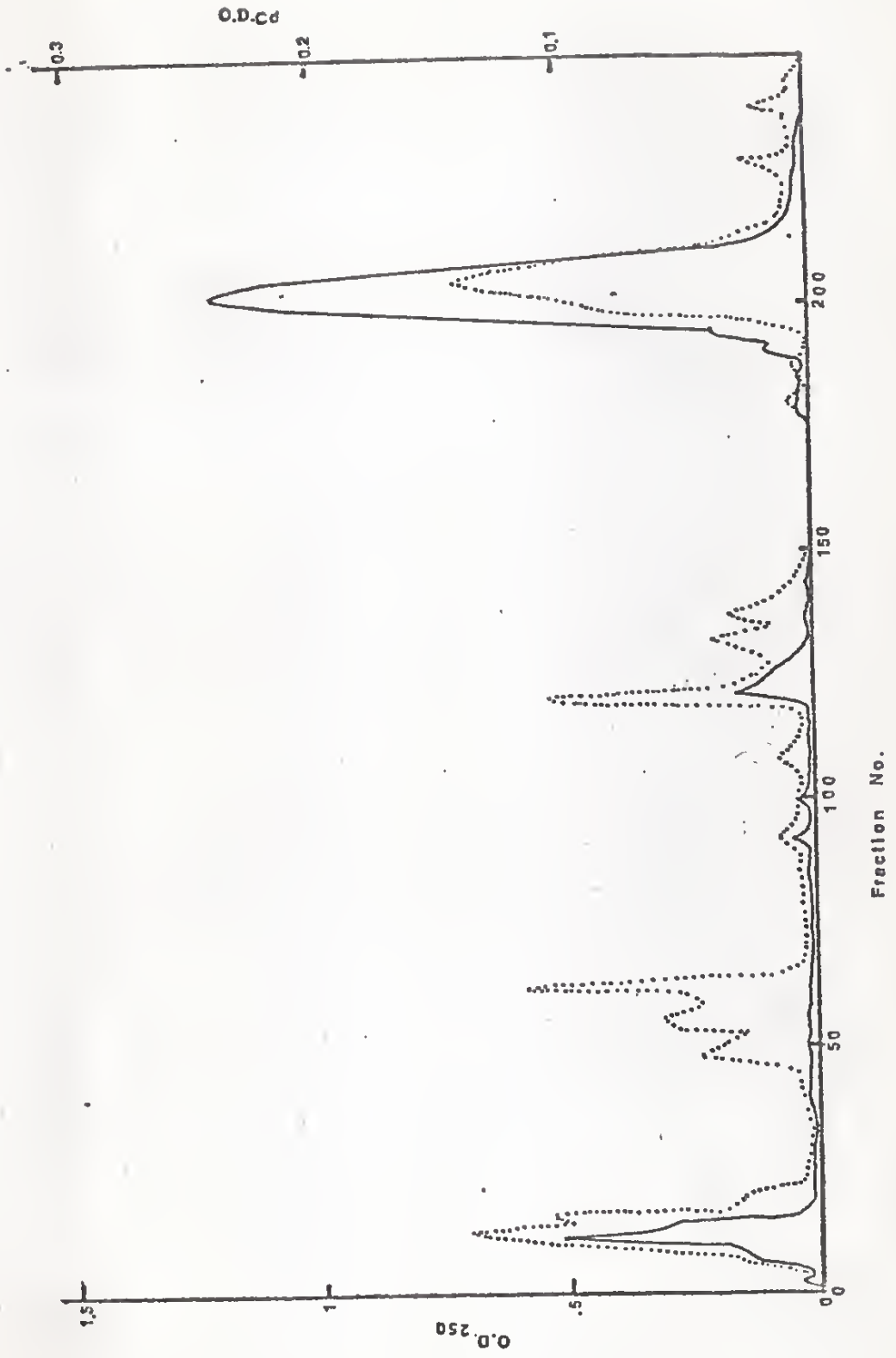

O.D. Cd
.2
.1
200
150
100
50
0
Fraction No.
O.D.250
2
1
0

FIGURE 17. DEAE SEPHADEX·A-25 CHROMATOGRAPHY OF PARTIALLY PURIFIED MT-2

MT-2 was eluted from the column with 1,000 ml of 0.03 M Tris-0.0075 M HCl, pH 8.6 followed by 1,600 ml of 0.06 M Tris-0.015 M HCl, pH 8.6. Ten ml fractions were collected.

----------absorbance of cadmium measured by atomic absorption spectrophotometer

————————250 nm absorbance


O.D.Cd
0.3
0.2
0.1
O.D.250
1.5
1
.5
0
0
50
100
150
200
Fraction No.

The homogeneity of purified MT-1 and MT-2 was shown by electrophoresis. The scanned gel profiles are shown in Figures18 and 19. Only one band was observed on the polyacrylamide gel for both MT-1 and MT-2, but MT-1 and MT-2 had different electrophoretic mobilities. MT-2 moved faster towards the anode than MT-1.

By using a calibrated Sephadex G-75 column, MT-1 and MT-2 were shown to have very similar molecular weight. Employing molecular weight markers, e.g. ovalbumin, lysozyme, ribonuclease, ACTH, and glucagon, the molecular weights of both MT-1 and MT-2 were shown to be in the proximity of 6,800 daltons. This molecular weight agreed with the minimum molecular weight estimated from amino acid analysis data.

The amino acid compositions of the two forms of metallothionein are shown in Table 16. The values found were comparable to that reported by Bühler and Kägi(93), except that histidine was detected in our preparation. The Durrum Amino Acid Analyzer model D-500 using o-phthalicdicarbonaldehyde reagent does not detect proline. From the Table, the most distinctive common features for MT-1 and MT-2 were the abundance of cysteine residues and the complete absence of aromatic amino acids and arginine. There were a number of differences between MT-1 and MT-2. MT-1 contained more valine and lysine, and a small quantity of leucine was also present. MT-2 had no leucine, and had more cysteine, aspartic acid, and alanine than MT-1. Determination of sulfhydryl groups in MT-1 and MT-2 (18.8 mole/mole MT-1 and 19.6 mole/mole MT-2) by the Ellman's reagent and comparison with the cysteic acid data showed that disulfide bonds are probably absent in the 2 proteins.

The metal content of the proteins is shown in Table 17. MT-2 contained more metal than MT-1. Both proteins contained zinc, cadmium, and copper, but the relative amount of each element present was different. Zinc was

FIGURE 18. POLYACRYLAMIDE GEL ELECTROPHORESIS SCANNED GEL PATTERN OF PURIFIED MT-1

Distance was measured from top of gel.
Gel was scanned at 550 nm.

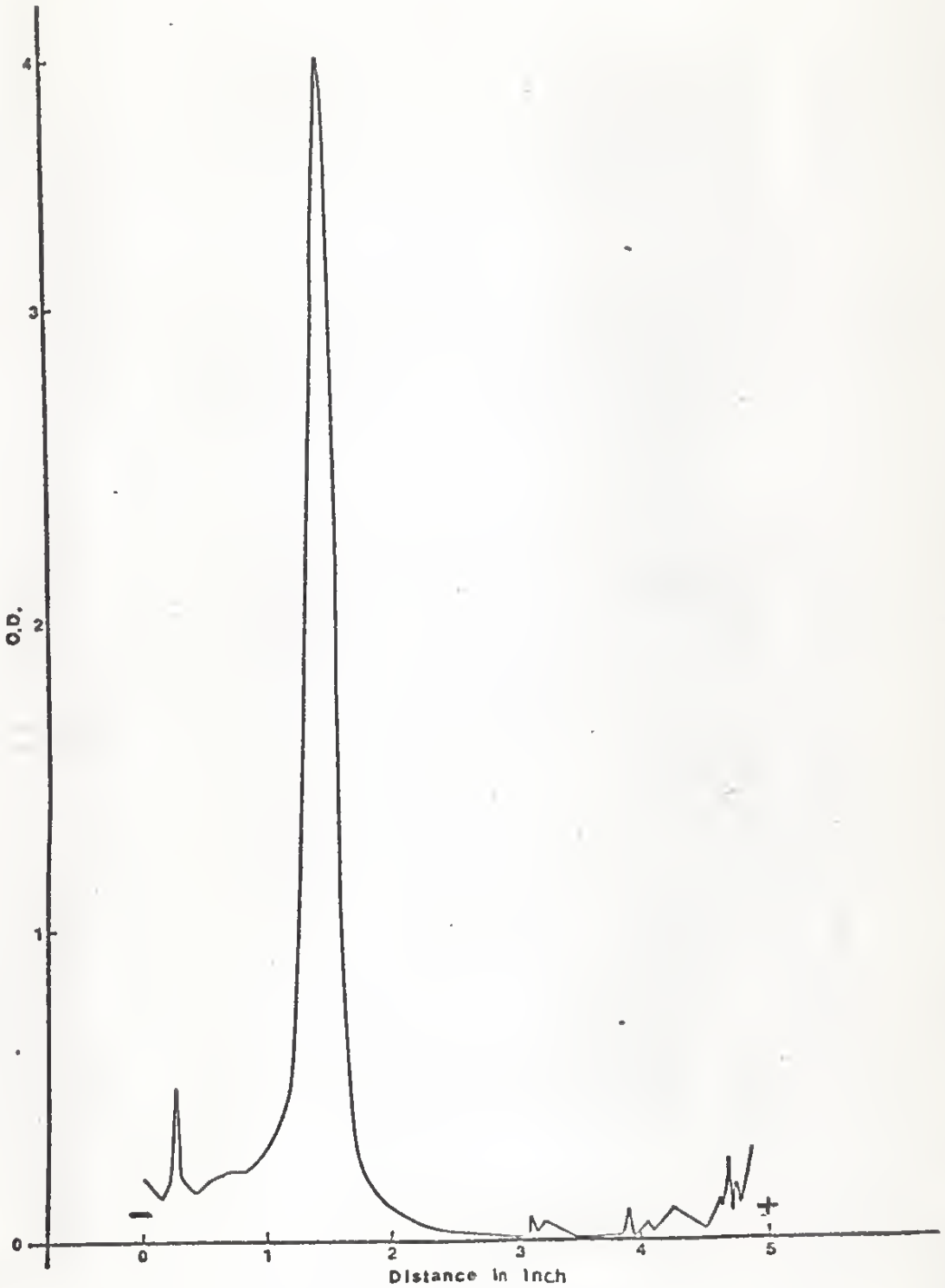

O.D.
4
3
2
1
0
0
1
2
3
4
5
Distance in Inch
−
+

FIGURE 19. POLYACRYLAMIDE GEL ELECTROPHORESIS SCANNED GEL PATTERN OF PURIFIED MT-2

Distance was measured from top of gel.
Gel was scanned at 550 nm.

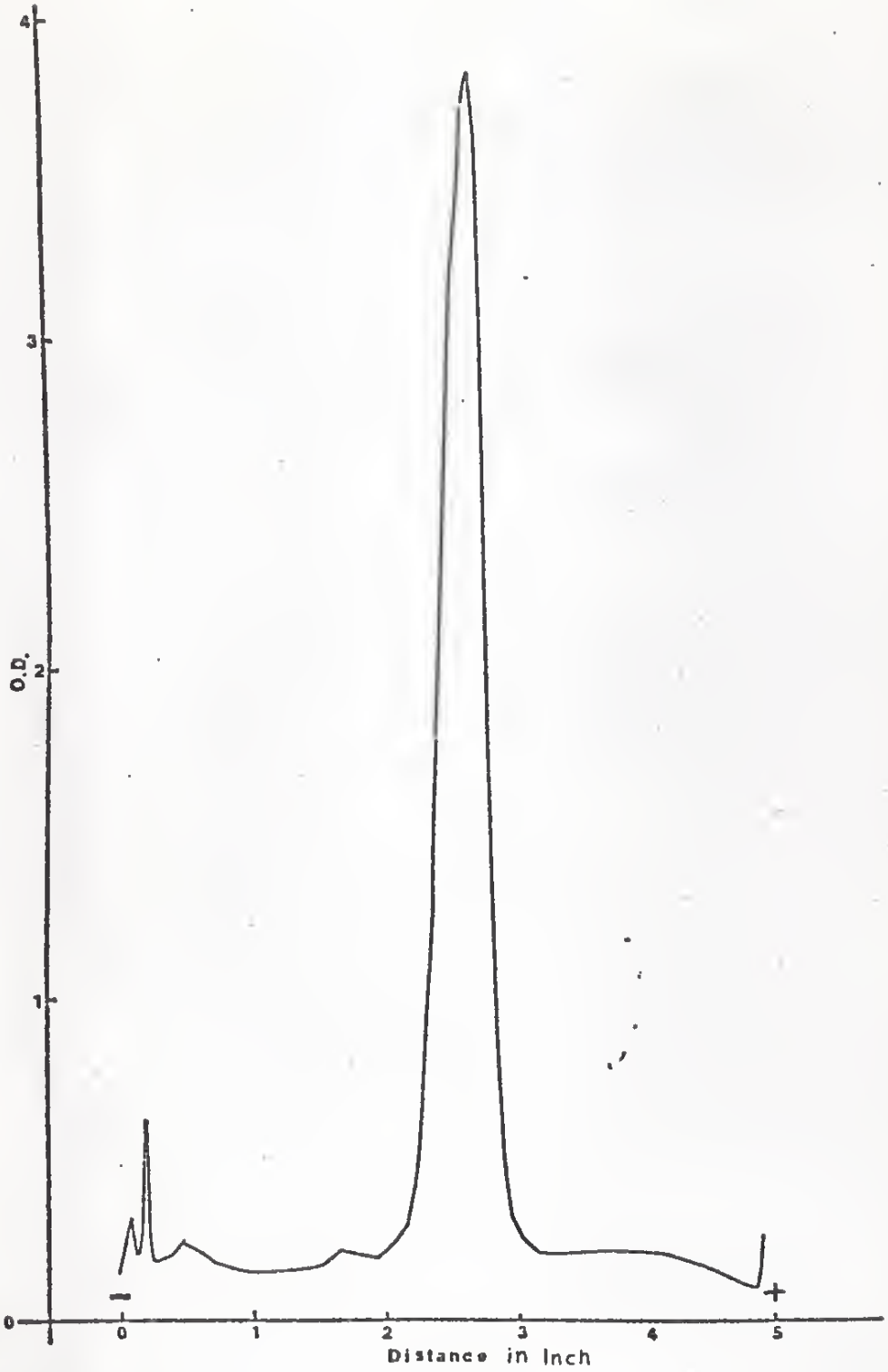

O.D.
4
3
2
1
0
0
1
2
3
4
5
Distance in Inch
−
+

TABLE 16

AMINO ACID COMPOSITIONS OF HUMAN LIVER [a, b] METALLOTHIONEINS

Amino Acids	MT-1 Number of Residue	MT-1 Nearest Integral Number	MT-2 Number of Residue	MT-2 Nearest Integral Number
Cysteic Acid	18.3	18	22.4	22
Asp	3.1	3	3.8	4
Meth. Sulfone	0.9	1	1.1	1
Thr	1.9	2	1.9	2
Ser	7.7	8	8.2	8
Glu	2.7	3	2.8	3
Gly	4.9	5	5.1	5
Ala	6.2	6	6.9	7
Val	2.0	2	0.9	1
Gle	1.1	1	1.2	1
Leu	0.40	-	0.0	-
Tyr	0.0	-	0.0	-
Phen	0.0	-	0.0	-
Hist	7.5	8	2.7	3
Lys	8.1	8	6.7	7
Avg	0.0	-	0.0	-
Tryp	0.0	-	0.0	-
Total	64.8	65.0	63.7	64.0

a. values are average of 3 analyses

b. proline is not determined by our machine

TABLE 17

METAL CONTENT OF HUMAN LIVER METALLOTHIONEINS[a]

	MT-1	MT-2
Zn g-atom/mole protein	6.59	9.01
Cd g-atom/mole protein	0.13	0.17
Cu g-atom/mole protein	0.35	0.08
Total g-atom metal/mole protein	7.07	9.25

a. Molecular weights of MT-1 and MT-2 are 6,800 daltons

the predominant element in both proteins. The second abundant element in MT-1 was copper, but in MT-2 was cadmium. Comparing the amount of metals with the quantity of mercaptide groups in both proteins, there would be 2.5 cysteinyl groups available per metal atom.

Purified metallothioneins were only isolated from normal livers due to the limited availability of MKHD liver. However, even in their crude forms MKHD hepatic metallothioneins demonstrated significant differences from normal hepatic metallothioneins. The most obvious changes (Table 15) are the great reductions in the sulfhydryl content and metal content in MKHD metallothioneins. Both MT-1 and MT-2 in MKHD liver had fewer sulfhydryl groups compared to the corresponding species from normal liver. However, the observation that MT-1 had more sulfhydryl groups than MT-2 was maintained in both normal and MKHD livers. Metal content was about 8-fold decreased in MKHD metallothioneins. Relative amounts of cadmium, copper, and zinc in the MKHD metallothioneins were also changed. In MT-1 and MT-2 cadmium demonstrated the least change. Even though it is inappropriate to extrapolate too much from the observations on the crude proteins, there were changes in the properties of both MT-1 and MT-2 in MKHD. The decreased metal content in MKHD metallothioneins might be due to a decrease in the sulfhydryl content. A decrease in sulfhydryl contents of both MT-1 and MT-2 was also consistant with the supposition deduced from the metal induction experiments that MKHD metallothionein appeared to have a smaller molecular weight than normal metallothionein.

Metal-Binding Studies

Inducibility of metallothionein in MKHD cultured fibroblasts was shown to be normal in the metal induction experiments described previously. Analysis of partially purified metallothioneins from MKHD liver reveals a

change in the metal content. Next we decided to investigate whether there is any change in the metal-binding parameter of the MKHD metallothioneins. Due to the limited availability of MKHD metallothioneins a complete Scatchard plot (206) is impossible. Besides, since the metallothioneins prepared are not in their very pure form, any attempt to determine the absolute number and association constants of their binding sites is invalid. An experiment was designed to compare the metal-binding properties of normal and MKHD metallothioneins. The affinity of a protein for metal can be expressed in the readiness of exchangeability of the metal with exogenous agents. Metals which are readily exchangeable are thought to be loosely bound by the protein. Results of the ^{64}Cu exchange experiment performed on normal and MKHD metallothioneins are presented in Table 18. MKHD MT-1 and MT-2 were found to bind more ^{64}Cu than normal MT-1 and MT-2. The ratio of bound ^{64}Cu between MKHD and normal in MT-1 is 1.7 and in MT-2 is 3.4. This increased ^{64}Cu binding of the MKHD metallothionein implies an increase in exchangeability of the bound copper compared with normal metallothionein. This in turn implies a decrease in copper affinity in MKHD metallothionein. There is also another explanation. Two kinds of copper binding sites are present in metallothioneins, one with high and the other with low affinity for copper. During preparation of the metallothioneins copper ions were removed from the low affinity sites. Thus, the final form of metallothioneins prepared would have the high affinity sites still occupied by copper, while the low affinity sites were unoccupied. Binding of ^{64}Cu occurred in the unoccupied sites. Increase in binding of ^{64}Cu is due to an increase in the number of the unoccupied sites, which in turn implies a decrease in the affinity for copper in the whole protein. In Table 18, the ratio of ^{64}Cu bound to the

TABLE 18

COPPER-64 EXCHANGE EXPERIMENT WITH PARTIALLY PURIFIED HEPATIC METALLOTHIONEINS [a, b]

	Metal Uptake[c]		
	$10^{-5} \times {}^{64}Cu$ cpm/mg protein	${}^{64}Cu$ g-atom/ mole protein	${}^{64}Cu$ g-atom / Total Copper g-atom
Normal			
MT_1	1.58±0.27	0.0027	0.021
MT_2	3.12±0.65	0.0054	0.084
MKHD			
MT_1	2.69±0.57	0.0047	0.158
MT_2	10.66±1.58	0.0185	0.673

a. values are means ± standard deviations of 2 experiments

b. specific activity of ${}^{64}Cu$ is 175.8 mCi/mg-atom

c. conditions described in section Metal-Binding Studies

total copper present in the protein is computed in the last column. It is obvious that the MKHD MT-1 and MT-2, which have a higher ratio of ^{64}Cu to total copper, have a larger number of unoccupied sites. This implies that the MKHD metallothioneins have lower affinity for copper.

The cadmium binding properties of the metallothioneins were also examined. The results are presented in Table 19. MKHD metallothioneins demonstrated a decrease in ^{109}Cd binding. The ratio of ^{109}Cd binding in MKHD to that in normal is 0.16 for MT-1 and 0.46 for MT-2, implying that both MKHD MT-1 and MT-2 have higher affinity for cadmium. The ratio of ^{109}Cd bound to total cadmium in the protein is higher in normal MT-1 and in MKHD MT-2. Thus MKHD MT-1 has a decreased and MKHD MT-2 has an increased number of unoccupied sites for cadmium. The discrepancies in the cadmium affinity of MKHD MT-2 derived from the 2 potential interpretations of the observations imply that binding to unoccupied sites as well as exchange of cadmium occurred in metallothioneins during the reaction.

To further investigate the nature of cadmium binding in the metallothioneins, the dialyzed ^{109}Cd containing metallothionein solutions were passed through a Bio-Gel P10 column equilibrated with 0.1 M ammonium bicarbonate, pH 8.2. The chromatograms are shown in Figure 20. It is obvious that the majority of the ^{109}Cd label is bound to one main peak in all of the metallothionein preparations. The major peak in each chromatogram represents the metallothionein fraction. Since Bio-Gel chromatography would remove most of the metals from the low affinity sites in the protein the presence of bound ^{109}Cd to the metallothionein fractions implies that the binding of ^{109}Cd in the previous experiment was not entirely due to the presence of unoccupied sites. Exchange of ^{109}Cd also occurred in the high affinity sites. Metal analysis of the major peaks

TABLE 19

CADMIUM-109 EXCHANGE EXPERIMENT WITH PARTIALLY PURIFIED HEPATIC METALLOTHIONEINS [a, b]

	Metal Uptake[c]		
	$10^{-5} \times {}^{109}Cd$ cpm/mg protein	^{109}Cd mg atom/ mole protein	$\frac{^{109}Cd \text{ g-atom}}{\text{Total Cadmium g-atom}}$
Normal			
MT_1	5.54±0.33	0.0218	0.028
MT_2	3.36±0.98	0.0132	0.006
MKHD			
MT_1	0.88±0.02	0.0035	0.006
MT_2	1.54±0.30	0.0059	0.012

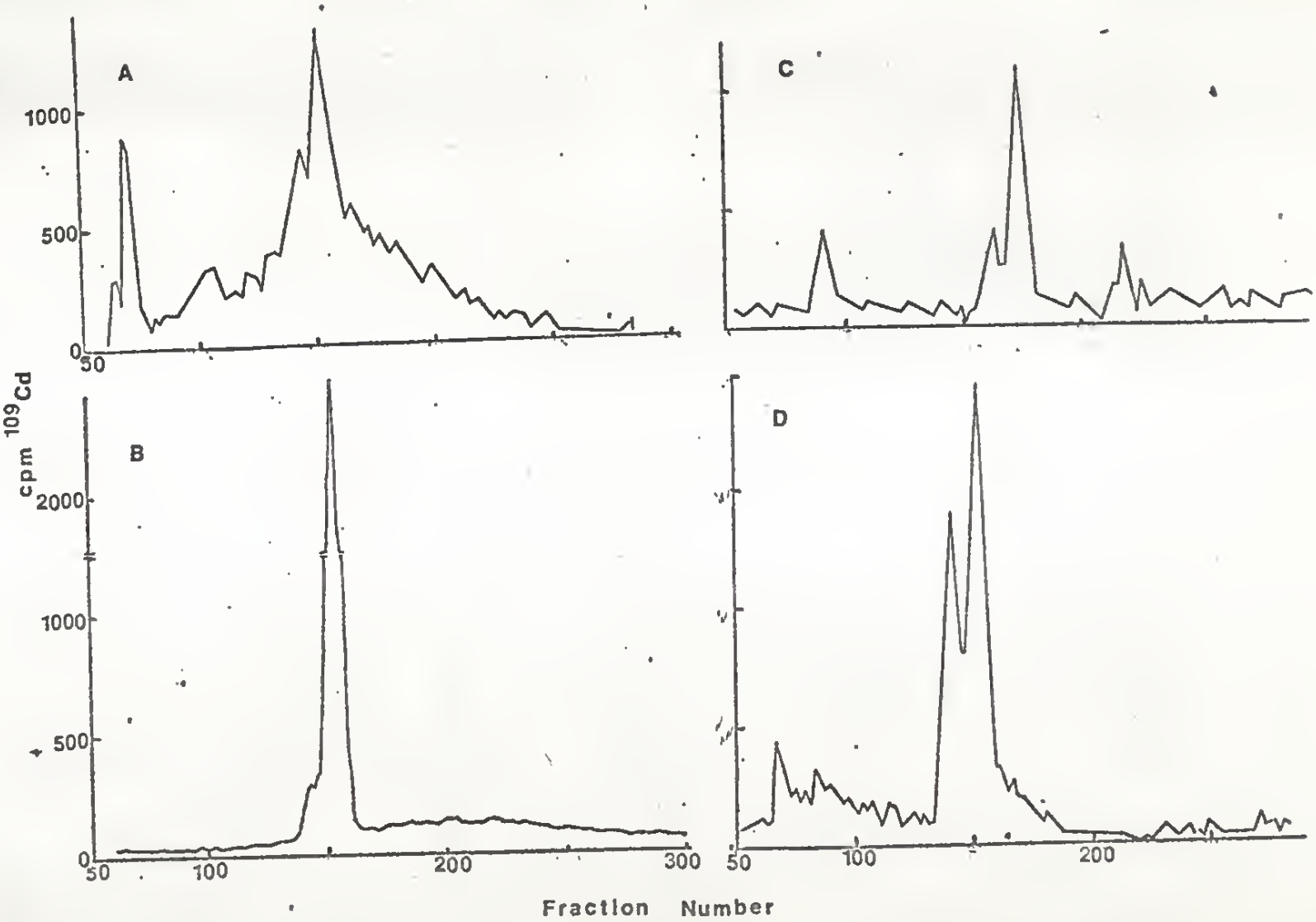
a. values are means ± standard deviations of 2 experiments

b. specific activity of ^{109}Cd is 77.56 mCi/μg-atom

c. conditions described in section Metal-Binding Studies

FIGURE 20. BIO-GEL P10 CHROMATOGRAPHY OF CADMIUM-109 LABELLED METALLOTHIONEINS

A. Normal MT-1
B. Normal MT-2
C. MKHD MT-1
D. MKHD MT-2


A
B
C
D
1000
500
0
50
2000
1000
500
0
50
100
200
300
50
100
200
cpm ^{109}Cd
Fraction Number

were performed and the results are presented in Table 20. The ratio of ^{109}Cd binding in MKHD to that in normal is 0.13 in MT-1 and 0.28 in MT-2. These results indicate an increase in cadmium affinity in both MKHD MT-1 and MT-2. Thus the observations made with crude preparations of metallothionein still apply when purer proteins were used. When the mobilities of the metallothioneins in Bio-Gel P 10 were compared, normal and MKHD MT-2 have the same mobility, indicating that they have the same molecular weight. MKHD MT-1, however, appeared to have a smaller molecular weight than normal MT-1 because it was eluted later.

The metal-binding experiments show that both MT-1 and MT-2 in MKHD have decreased copper affinity. There was also a change in the molecular weight of MKHD MT-1. The molecular weight of MKHD MT-2 appears to be similar to that of normal MT-2.

Sulfhydryl Affinity Chromatography

The sulfhydryl affinity chromatography technique to isolate metallothionein from liver homogenate was unsuccessful. There are several possible reasons. The sulfhydryl groups in metallothionein may have been oxidized to form disulfide bonds during the preparation of apo-metallothionein, making the apo-protein unable to attach itself to the resin. It is also possible that the interaction of the apo-protein sulfhydryl groups with the sulfhydryl deriative of the resin is not strong enough to displace thiopyridone, so that the apo-metallothionein was lost during washing after the coupling procedure. Another possible reason was that after treatment with 2-mercaptoethanol, cadmium was unable to bind the RS^- group. Hence the protein was not detected by monitoring cadmium content or 250 nm absorbance.

However, 3 peptides were separated after Sephadex G-10 chromatography. Each peptide gave a single peak on Sephadex G-50 chromatography indicating

TABLE 20

CADMIUM-109 ANALYSIS OF THE MAJOR PEAKS IN BIO-GEL P10 CHROMATOGRAPHY OF PARTIALLY PURIFIED HEPATIC METALLOTHIONEINS

	% recovery of ^{109}Cd counts in major peak	% recovery of protein in major peak	^{109}Cd µg-atom/ mole protein
Normal			
MT_1	45.9	70.2	15.90
MT_2	65.1	98.1	13.35
MKHD			
MT_1	53.7	85.4	1.99
MT_2	48.1	72.3	3.80

that they were homogeneous. The amino acid analysis of the peptides are presented in Table 21. All 3 peptides contained a large number of histidine residues. Peptide I, in addition, contained considerable amount of aspartic acid, leucine, alanine, glycine, valine and glutamic acid. It is interesting to know that under normal conditions histidine is the amino acid primarily involved in the formation of complexes with copper (59). Tyrosine was absent in any of the peptides and methionine, valine, and arginine were also absent from peptides II and III.

General Discussion

The previous discussion has indicated the presence of abnormal metallothioneins in MKHD liver.

Some of the roles played by metallothioneins are in intestinal absorption (10,81,141), and in intracellular transport of copper (10,104). There are at least 3 steps in the intestinal absorption of copper. The first step is in the uptake of copper from the lumen. Copper in digested foodstuff is transported through the intestinal mucosal membrane in complexed (6,10,43-46) and in ionic form (6,47).

Another step is the release of copper from the mucosal cells into plasma. Not all the copper transported into the mucosal cells passes into the bloodstream. Some remains bound to proteins in the mucosal cells (10), and some remains in the form in which it was transported across the membrane (207). By using everted sacs of hamster intestine, Crampton et al. (52) showed that the egress of copper from the intestinal wall is mainly unidirectional. In the plasma 93 % of the copper are bound to ceruloplasmin (8,208,209). The copper in this compartment is not in equilibrium with ionic copper *in vitro* and is not exchangeable *in vivo* (8,72). Most of the non-ceruloplasmin-bound copper is bound to albumin (8,53,208).

TABLE 21

AMINO ACID COMPOSITIONS OF PEPTIDES ISOLATED BY SULFHYDRYL AFFINITY CHROMATOGRAPHY

	Cys. Acid	Asp	Meth. Sulf.	Thr	Ser	Glu	Gly	Ala	Val	Ileu	Leu	Thr	Phen	Hist	Lys	Ave
Peptide I *	1.8	10.1	1.0	5.4	5.7	8.0	8.3	8.9	8.2	2.5	10.3	0	4.9	57.8	4.4	2.3
Peptide II	6.3	2.6	0	1.6	2.8	2.5	7.5	2.6	0	1.0	2.5	0	1.6	171.0	4.7	0
Peptide III	1.6	3.0	0	1.4	2.3	2.3	4.9	2.0	0	1	2.3	0	1.4	83.9	1.7	0

*Peptides are numbered according to the order of elution from sephadex G-10 column

This latter fraction is in equilibrium with tissue copper (53) and is considered to be the immediate transport form of copper in plasma (8,59, 62). A small fraction of copper in plasma is found to bind to amino acids, mainly histidine, glutamine and threonine (62,63).

The mechanism of the intermediate step, i.e. the intracellular transport of copper from the intestinal side to the serosal side within the mucosal cells, is unknown. Evans (10) hypothesized that metallothionein is responsible for carrying copper to the serosal side of the mucosal membrane.

Wilson's disease patients suffer from excess accumulation of copper in tissues. A defective metallothionein with higher than normal affinity for copper was postulated to be responsible for the disease (91).

The basic biochemical defect of Menkes kinky hair disease was postulated to be in the intestinal absorption of copper (145). More specifically experimental evidences hinted that the defect in copper absorption is in the transport within the mucosal and epithelial cells of the gut or across the membrane on the serosal aspect of these cells (155,165). Hunt (210), using an animal model for MKHD, also arrived at the same conclusion. Based on these observations the hypothesis that MKHD is caused by the absence or abnormality of metallothionein was put forth (162).

The results of this research seem to support the hypothesis that MKHD is caused, at least in part, by defective metallothionein. The abnormality in the metallothionein in MKHD is its reduced affinity for copper. A hypothesis is proposed to explain the mechanism of intracellular transport of copper within cultured fibroblasts, with the possibility that it also represents the in vivo copper absorption mechanism.

Metallothioneins are supposed to function in the intracellular transport of copper. MT-1 as indicated by its inherent higher copper

content, is probably mainly responsible for intracellular copper transport, while both MT-1 and MT-2 participate in cadmium transport. Copper and cadmium in the medium are present in ionic form as well as complexed with amino acids. The diffusion of ionic and complexed cations into cells is unidirectional. Inside the cell the metals combine with metallothionein. For outward transport from the cell binding to metallothionein is a necessary criterion. Metallothionein carrying metals moves to the cellular membrane. The metallothionein bound metals are then transferred directly or via a membrane carrier protein to an extracellular carrier, e.g. albumin in plasma. The scheme is depicted in Figure 21. In the Figure, MT-1-Cu, MT-2-Cu, MT-1-Cd, and MT-2-Cd are metallothionein metal complexes. The dotted lines represent pathways of secondary importance. The fate of MT-1-Cd and MT-2-Cd is unknown. Probably, they will remain in the cell and be discarded with the shedding of the epithelial cells, and thus prevent accumulation of harmful cadmium. The carrier in the Figure may be an extracellular copper carrier, or a membrane-bound mediator and an extracellular carrier for copper. Presumably, the primary defect in MKHD is a MT-1 with lower copper affinity. This will be manifested as an increased in retention of copper by the cells.

This hypothesis is consistant with all the observations made. The protective effect of metallothionein against metal poisoning is by binding the metal ions, thus preventing them from attacking other proteins (140). Metallothionein in MKHD cell with a lower affinity for copper will be less efficient in binding the copper which diffuses into the cell, resulting in a less efficient excretory mechanism. This explains the higher intracellular copper concentration of MKHD cells in basal medium, as well as the decreased tolerance to medium copper. This also explains

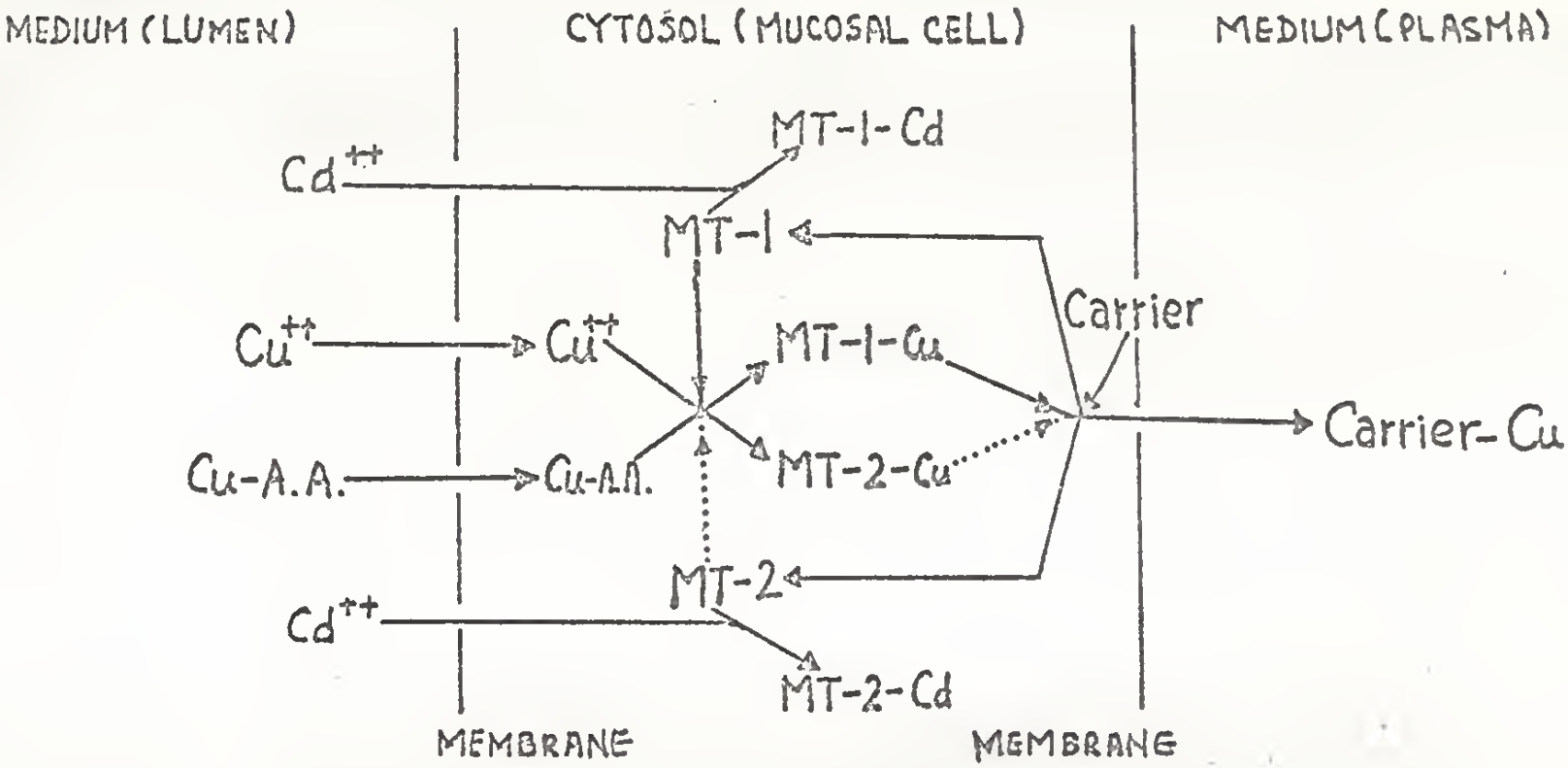


FIGURE 21. SCHEMATIC DIAGRAM ILLUSTRATING INTRACELLULAR TRANSPORT OF COPPER

Cu-A.A., Copper amino acid complex

————————major pathway
----------minor pathway

why MKHD cells need a higher intracellular copper concentration before the excretory mechanism can demonstrate any effect. Influx of copper, because it is independent of metallothionein, will not be affected in MKHD cells. Another manifestation of the decreased efficiency in excretion is the increased retention of copper by MKHD cells. The observation that in MKHD patients and in the mouse mutant brindles, which exhibits similar symptoms as MKHD, abnormally high accumulation of copper was found in duodenal mucosal cells after oral ingestion of copper, is also accounted for by a similar argument. Defective utilization of intravenous copper in MKHD patients is also understandable as the metallothionein has less capability to bind with copper transferred from the copper carrier in the bloodstream.

The increased cadmium affinity of the MKHD metallothioneins can be accounted for based en these postulations. Because of higher affinity of cadmium, the MKHD metallothioneins can bind more cadmium than normal, thus raising the tolerance level of cadmium of the MKHD cells.

The hypothesis just presented partly clarifies the missing link in intestinal absorption of copper. The transport role of metallothionein in copper metabolism is suggested. The unique behavior of MKHD cells in culture indicates the potential use of it as an in vitro model for studying MKHD. In addition, the high intracellular copper concentration of the MKHD cell and its response towards copper treatment provide an in vitro genetic marker for MKHD. This genetic marker may be useful to identify carriers of the defective gene, and also provides a basis upon which prenatal diagnosis and a rational mode of therapy can be developed.

Even though this dissertation provides enough evidence to support the proposed hypothesis, several further experiments will provide more

conclusive evidence. The data indicate that there is a change in the metal-binding affinity of the MKHD metallothioneins. Thus, the most informative experiments will be to determine the exact binding constants and number of binding sites in both MKHD and normal metallothioneins by Scatchard plots. A change in affinity can be due to a change in the primary structure of the metallothioneins. This can be determined by sequencing the mutant metallothionein. Preparation of antibodies against normal metallothioneins will also be useful as they may be used to examine any change in the tertiary structure of the MKHD metallothioneins. They may also be used to prepare an immunoaffinity column for rapid preparation of pure metallothionein. Even though metallothioneins were proved to be inducible in both normal and MKHD cells, there are no experimental data concerning the quantities of metallothioneins present. Antibodies can be used to quantitate metallothioneins in normal and MKHD cells. One question concerning preparation of antibodies is the ease of preparing pure metallothioneins. Sulfhydryl affinity chromatography is probably the most specific way to prepare pure metallothionein.

Since in MKHD not only intestinal absorption but also utilization of intravenuos copper is defective, it is worthwhile to study the metallothioneins from other tissues besides liver, e.g. brain, kidney, intestine etc. to determine whether the metallothioneins are homologous. The mouse mutant brindles which exhibits symptoms similar to MKHD in human should also be studied to see whether it can serve as an in vivo model for MKHD. To begin with, the behavior of the mouse mutant fibroblasts in culture can be compared and contrasted with the MKHD cultured fibroblasts. Wilson's disease is another disease which can be studied in the same manner.

There is one point which needs further elaboration for the proposed hypothesis, namely. the interaction between metallothionein and the extracellular carrier. The first question to be answered is whether this process involves any membrane-bound mediator. Metallothionein was shown to be present in the cytosol. Thus, the transfer of copper from metallothionein to the extracellular carrier must occur on or near the plasma membrane. The second question in need of answer will be whether there is any change in the plasma membrane of MKHD cell which will interfer with the transfer process. Examination of the cell membrane of MKHD cell under electron microscope may be helpful in the elucidation of the role of the plasma membrane.

SUMMARY

Possibility of the cultured fibroblasts of affected infants of MKHD to serve as an in vitro model of the disease was investigated. Using fibroblasts from 6 patients with MKHD studies demonstrated abnormal uptake and efflux of copper in the mutant cells. Physicochemical studies, using post-mortem tissues from one of these patients, implicated structurally abnormal liver metallothioneins having a decreased affinity for copper and an increased affinity for cadmium.

Normal and MKHD cultured fibroblasts exhibited different cytotoxic levels of copper. MKHD fibroblasts demonstrated morphological changes at medium copper concentrations between 15 and 20 µg/ml. Normal fibroblasts remained viable until the medium copper concentration reached 30 µg/ml. In basal culture medium, the intracellular copper concentration of MKHD fibroblasts was 310 ± 64.4 ng/mg cell protein compared with 108 ± 20.6 ng/mg cell protein in normal cultures. At medium copper concentrations less than 20 µg/ml, the intracellular copper of MKHD fibroblasts was twice that of normal fibroblasts. At concentrations from 20 to 45 µg/ml (approximately 30 times physiological concentration) the intracellular copper concentration of normal cells approached that of MKHD cells. MKHD cells began dying when the intracellular copper concentration was between 800 and 970 ng/mg cell protein, which was about 3 times the base level, while death of normal cells occurred only at concentrations above 1,500 ng/mg cell protein (15 times the base level). These observations suggested the presence of a

regulatory mechanism for intracellular copper in normal fibroblasts at medium copper concentrations below 30 μg/ml, which was defective in MKHD fibroblasts at all concentrations.

In basal medium, intracellular cadmium concentration of both MKHD and normal fibroblasts was about 23.5 ng/mg cell protein. The intracellular cadmium concentration of MKHD cells increased more than that of normal cells at medium cadmium concentrations above 0.1 μg/ml. MKHD cell death occurred at an intracellular cadmium concentration 2 to 3 times that for normal cells. These observations implied an increased uptake of cadmium by MKHD cells.

To further clarify the mechanism of metal uptake in cultured fibroblasts, incorporation studies were done with both ^{64}Cu and ^{109}Cd at 37°C over a period of 10 hours. Uptake rate of ^{64}Cu in the first hour was about 3 times greater in MKHD cells than in normal cells, while that of ^{109}Cd was about the same in both types of cells. Subsequent uptake of both radionuclides was higher in MKHD cells than in normal cells. Uptake of ^{64}Cu in both types of cells was also enhanced by the presence of cadmium. These observations indicated that the influxes of ^{64}Cu and ^{109}Cd were not impaired in MKHD cells.

Effluxes of ^{64}Cu and ^{109}Cd in both MKHD and normal cells were studied by pulse labelling the cells with the radionuclides for 20 hours and chased with non-radioactive medium. At both 37°C and 4°C, MKHD cells demonstrated a higher retention of both radionuclides than normal cells, indicating an impairment of efflux of both metals in MKHD cells. Lowering the temperature did not increase the percent retention of either radionuclide in the 2 types of cells, implying that the efflux processes were not energy requiring.

Inducibility of metallothioneins in cultured fibroblasts by copper and cadmium was investigated utilizing L-(^{35}S) cysteine. No metallothionein was induced when the cells were incubated with ^{64}Cu. ^{109}Cd was found to induce metallothionein synthesis in both normal and MKHD cells. The onset of synthesis was found to be between the seventh and the tenth hour of incubation. In addition, the metallothionein induced by cadmium in MKHD cells appeared to have smaller molecular weight than that in normal cells.

Partially purified metallothioneins (MT-1 and MT-2) from 1 MKHD and 2 normal livers were analyzed. MKHD metallothioneins showed a reduced cadmium, copper, and zinc content. Furthermore, quantification of sulfhydryl groups in the metallothioneins using Ellman's reagent demonstrated a reduced content in both MKHD MT-1 and MT-2. This implicated a structural abnormality in MKHD metallothioneins which was reflected in a marked alteration in their physiocochemical properties. Isotope exchange studies, carried out by incubating native metallothioneins with ^{64}Cu or ^{109}Cd, demonstrated a decreased affinity for copper and an increased affinity for cadmium in both MKHD metallothioneins.

Thus, intracellular copper and cadmium content, cytotoxic levels of copper and cadmium, in vitro fibroblast uptake and efflux, and the physicochemical analysis of MKHD metallothioneins imply an abnormality in MKHD fibroblast copper and cadmium transport, which is associated with structural abnormalities in metallothioneins, reduced affinity for copper and increased affinity for cadmium. A hypothesis is proposed to explain the function of metallothionein in copper metabolism and its etiological role in MKHD. Metallothioneins, especially MT-1, function as carrier for incoming copper ions by binding and carrying them to the

sites of excretion. At these sites, which may be intracellular or on the cell membrane, metallothioneins release their copper to the membrane-bound or extracellular carrier. Abnormality in MKID resides in the decreased affinity for copper of the metallothioneins which are unable to bind the incoming copper and hence fail to help excretion of copper from the cells

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Wai-Yee Chan was born on April 28, 1950, in Canton of Kwangtung, China. He graduated from secondary school in 1968 and Upper Form Six in 1970 from Queen's College, Hong Kong.

In 1974, he graduated from the Chinese University of Hong Kong with a major in chemistry and a minor in biochemistry. He was awarded the degree of Bachelor of Science with Honours, 1st class. During the first 2 years of his undergraduate education he worked as a research student under Professor Lin Ma of the Biochemistry Department of Chinese University of Hong Kong on developing methodologies for routine determination of body-fluid glucose and serum albumin in clinical biochemistry laboratories. Part of the research was done in the Division of Clinical Pathology of the Caritas Medical Center of Hong Kong. During his junior and senior years, he worked under Dr. Walter K. K. Ho of the Radioisotope Unit of Hong Kong University on topics concerning human serum lipoproteins. Topics investigated included "Human serum lipoprotein analysis-an electrophoretic approach," "Serum lipid and lipoprotein levels in normal Chinese," "The influence of dietary habit, body weight, exercise and a familial record of coronary heart disease on serum lipid and lipoprotein levels," and "Heterogeneity of human serum very low density lipoproteins." In 1974, he entered the Department of Biochemistry and Molecular Biology of the University of Florida.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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